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(57) Abstract

Targetable diagnostic and/or therapeutically active agents, e.g. ultrasound contrast agents, having reporters comprising gas–filled microbubbles stabilised by monolayers of film–forming surfactants, the reporter being coupled or linked to at least one vector.

Improvements in or relating to diagnostic/therapeutic agents

5   This invention relates to diagnostic and/or
therapeutically active agents, more particularly to
diagnostic and/or therapeutically active agents
incorporating moieties which interact with or have
affinity for sites and/or structures within the body so
10   that diagnostic imaging and/or therapy of particular
locations within the body may be enhanced. Of
particular interest are diagnostic agents for use in
ultrasound imaging, which are hereinafter referred to as
targeted ultrasound contrast agents.

15   It is well known that ultrasound imaging comprises
a potentially valuable diagnostic tool, for example in
studies of the vascular system, particularly in
cardiography, and of tissue microvasculature. A variety
of contrast agents has been proposed to enhance the
20   acoustic images so obtained, including suspensions of
solid particles, emulsified liquid droplets, gas bubbles
and encapsulated gases or liquids. It is generally
accepted that low density contrast agents which are
easily compressible are particularly efficient in terms
25   of the acoustic backscatter they generate, and
considerable interest has therefore been shown in the
preparation of gas-containing and gas-generating
systems.

Gas-containing contrast media are also known to be
30   effective in magnetic resonance (MR) imaging, e.g. as
susceptibility contrast agents which will act to reduce
MR signal intensity. Oxygen-containing contrast media
also represent potentially useful paramagnetic MR
contrast agents.

35   Furthermore, in the field of X-ray imaging it has
been observed that gases such as carbon dioxide may be
used as negative oral contrast agents or intravascular

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contrast agents.

The use of radioactive gases, e.g. radioactive isotopes of inert gases such as xenon, has also been proposed in scintigraphy, for example for blood pool imaging.

Targeted ultrasound contrast agents may be regarded as comprising (i) a reporter moiety capable of interacting with ultrasound irradiation to generate a detectable signal; (ii) one or more vectors having affinity for particular target sites and/or structures within the body, e.g. for specific cells or areas of pathology; and (iii) one or more linkers connecting said reporter and vector(s), in the event that these are not directly joined.

The molecules and/or structure to which the agent is intended to bind will hereinafter be referred to as the target. In order to obtain specific imaging of or a therapeutic effect at a selected region/structure in the body the target must be present and available in this region/structure. Ideally it will be expressed only in the region of interest, but usually will also be present at other locations in the body, creating possible background problems. The target may either be a defined molecular species (i.e. a target molecule) or an unknown molecule or more complex structure (i.e. a target structure) which is present in the area to be imaged and/or treated, and is able to bind specifically or selectively to a given vector molecule.

The vector is attached or linked to the reporter moiety in order to bind these moieties to the region/structure to be imaged and/or treated. The vector may bind specifically to a chosen target, or it may bind only selectively, having affinty also for a limited number of other molecules/structures, again creating possible background problems.

There is a limited body of prior art relating to targeted ultrasound contrast agents. Thus, for example,

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US-A-5531980 is directed to systems in which the
reporter comprises an aqueous suspension of air or gas
microbubbles stabilised by one or more film-forming
surfactants present at least partially in lamellar or
laminar form, said surfactant(s) being bound to one or
more vectors comprising "bioactive species designed for
specific targeting purposes". It is stated that the
microbubbles are not directly encapsulated by surfactant
material but rather that this is incorporated in liquid-
filled liposomes which stabilise the microbubbles. It
will be appreciated that lamellar or laminar surfactant
material such as phospholipids present in such liposomes
will inevitably be present in the form of one or more
lipid bilayers with the lipophilic tails "back-to-back"
and the hydrophilic heads both inside and outside (see
e.g. Schneider, M. on "Liposomes as drug carriers: 10
years of research" in *Drug targeting, Nyon, Switzerland,
3-5 October 1984*, Buri, P. and Gumma, A. (Ed), Elsevier,
Amsterdam 1984).

EP-A-0727225 describes targeted ultrasound
contrast agents in which the reporter comprises a
chemical having a sufficient vapour pressure such that a
proportion of it is a gas at the body temperature of the
subject. This chemical is associated with a surfactant
or albumin carrier which includes a protein-, peptide-
or carbohydrate-based cell adhesion molecule ligand as
vector. The reporter moieties in such contrast agents
correspond to the phase shift colloid systems described
in WO-A-9416739; it is now recognised that
administration of such phase shift colloids may lead to
generation of microbubbles which grow uncontrollably,
possibly to the extent where they cause potentially
dangerous embolisation of, for example, the myocardial
vasculature and brain (see e.g. Schwarz, *Advances in
Echo-Contrast [1994(3)]*, pp 48-49).

WO-A-9320802 proposes that tissue-specific
ultrasonic image enhancement may be achieved using

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acoustically reflective oligolamellar liposomes
conjugated to tissue-specific ligands such as
antibodies, peptides, lectins etc. The liposomes are
deliberately chosen to be devoid of gas and so will not
have the advantageous echogenic properties of gas-based
ultrasound contrast agents. Further references to this
technology, e.g. in targeting to fibrin, thrombi and
atherosclerotic areas are found in publications by
Alkanonyuksel, H. *et al.* in *J. Pharm. Sci.* (1996) **85(5)**,
486-490; *J. Am. Coll. Cardiol.* (1996) **27(2) Suppl A**,
298A; and *Circulation*, *68 Sci. Sessions*, Anaheim 13-16
November 1995.

　　　There is also a number of publications concerning
ultrasound contrast agents which refer in passing to
possible use of monoclonal antibodies as vectors without
giving significant practical detail and/or to reporters
comprising materials which may be taken up by the
reticuloendothelial system and thereby permit image
enhancement of organs such as the liver - see, for
example WO-A-9300933, WO-A-9401140, WO-A-9408627, WO-A-
9428874, US-A-5088499, US-A-5348016 and US-A-5469854.

　　　The present invention is based on the finding that
gas-filled microbubbles stabilised by monolayers of
film-forming surfactant material are particularly useful
reporters in targeted diagnostic and/or therapeutic
agents. Thus, for example, the flexibility and
deformability of such thin monolayer membranes
substantially enhances the echogenicity of such
reporters relative to liposome systems containing lipid
bilayers or multiples of such bilayers. This may permit
the use of very low doses of the reporter material to
achieve high ultrasound contrast efficacy, with
consequent safety benefits.

　　　Thus according to one aspect of the present
invention there is provided a targetable diagnostic
and/or therapeutically active agent, e.g. an ultrasound
contrast agent, comprising a suspension in an aqueous

carrier liquid, e.g. an injectable carrier liquid, of a
reporter comprising gas-filled microbubbles stabilised
by monolayers of film-forming surfactant material, said
agent further comprising at least one vector.

5   The term "monolayer" is used herein to denote that
the amphiphilic surfactant moieties form monolayer films
or membranes similar to so-called Langmuir-Blodgett
films at the gas-liquid interfaces, with the lipophilic
parts of the amphiphiles aligning towards the gas phase

10   and the hydrophilic parts interacting with the water
phase.
       As indicated in WO-A-9729783, it is believed that
electrostatic repulsion between charged phospholipid
membranes encourages the formation of stable and

15   stabilising monolayers at microbubble-carrier liquid
interfaces. The flexibility and deformability of such
thin membranes are believed to enhance the echogenicity
of products according to the invention disclosed therein
relative to gas-filled liposomes comprising one or more

20   lipid bilayers. The amount of phospholipid used to
stabilise such microbubble-containing aqueous
suspensions may be as low as that necessary for
formation of single monolayers of surfactant around each
gas microbubble, the resulting film-like structure

25   stabilising the microbubbles against collapse or
coalescence. Microbubbles with a liposome-like
surfactant bilayer are believed not to be obtained when
such low phospholipid concentrations are used.
       One advantageous embodiment of the invention is

30   based on the additional finding that limited adhesion to
targets is a highly useful property of diagnostic and/or
therapeutically active agents, which property may be
achieved using vectors giving temporary retention rather
than fixed adhesion to a target. Thus such agents,

35   rather than being fixedly retained at specific sites,
may for example effectively exhibit a form of retarded
flow along the vascular endothelium by virtue of their

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transient interactions with endothelial cells. Such
agents may thus become concentrated on the walls of
blood vessels, in the case of ultrasound contrast agents
providing enhanced echogenicity thereof relative to the
5   bulk of the bloodstream, which is devoid of anatomical
features. They therefore may permit enhanced imaging of
the capillary system, including the microvasculature,
and so may facilitate distinction between normal and
inadequately perfused tissue, e.g. in the heart, and may
10   also be useful in visualising structures such as Kupffer
cells, thrombi and atherosclerotic lesions or for
visualising neo-vascularised and inflamed tissue areas.
The present invention is particularly suited to imaging
changes which occur in normal blood vessels situated in
15   areas of tissue necrosis.

In a further embodiment of the present invention,
one or more vectors may be attached to or included
within the reporter in a manner such that the vectors
are not readily exposed to the target or target
20   receptors. Increased tissue specificity may therefore
be achieved by applying an additional process to expose
the vectors, for example by exposing the agent after
administration to external ultrasound so as to modify
the diffusibility of the moieties containing the
25   vectors.

Any biocompatible gas may be present in the
reporter, the term "gas" as used herein including any
substances (including mixtures) substantially or
completely in gaseous (including vapour) form at the
30   normal human body temperature of 37°C. The gas may
thus, for example, comprise air; nitrogen; oxygen;
carbon dioxide; hydrogen; an inert gas such as helium,
argon, xenon or krypton; a sulphur fluoride such as
sulphur hexafluoride, disulphur decafluoride or
35   trifluoromethylsulphur pentafluoride; selenium
hexafluoride; an optionally halogenated silane such as
methylsilane or dimethylsilane; a low molecular weight

hydrocarbon (e.g. containing up to 7 carbon atoms), for
example an alkane such as methane, ethane, a propane, a
butane or a pentane, a cycloalkane such as cyclopropane,
cyclobutane or cyclopentane, an alkene such as ethylene,
5   propene, propadiene or a butene, or an alkyne such as
acetylene or propyne; an ether such as dimethyl ether; a
ketone; an ester; a halogenated low molecular weight
hydrocarbon (e.g. containing up to 7 carbon atoms); or a
mixture of any of the foregoing. Advantageously at
10   least some of the halogen atoms in halogenated gases are
fluorine atoms; thus biocompatible halogenated
hydrocarbon gases may, for example, be selected from
bromochlorodifluoromethane, chlorodifluoromethane,
dichlorodifluoromethane, bromotrifluoromethane,
15   chlorotrifluoromethane, chloropentafluoroethane,
dichlorotetrafluoroethane, chlorotrifluoroethylene,
fluoroethylene, ethylfluoride, 1,1-difluoroethane and
perfluorocarbons, e.g. perfluoroalkanes such as
perfluoromethane, perfluoroethane, perfluoropropanes,
20   perfluorobutanes (e.g. perfluoro-n-butane, optionally in
admixture with other isomers such as perfluoro-iso-
butane), perfluoropentanes, perfluorohexanes and
perfluoroheptanes; perfluoroalkenes such as
perfluoropropene, perfluorobutenes (e.g. perfluorobut-2-
25   ene) and perfluorobutadiene; perfluoroalkynes such as
perfluorobut-2-yne; and perfluorocycloalkanes such as
perfluorocyclobutane, perfluoromethylcyclobutane,
perfluorodimethylcyclobutanes, perfluorotrimethyl-
cyclobutanes, perfluorocyclopentane, perfluoromethyl-
30   cyclopentane, perfluorodimethylcyclopentanes,
perfluorocyclohexane, perfluoromethylcyclohexane and
perfluorocycloheptane. Other halogenated gases include
methyl chloride, fluorinated (e.g. perfluorinated)
ketones such as perfluoroacetone and fluorinated (e.g.
35   perfluorinated) ethers such as perfluorodiethyl ether.
The use of perfluorinated gases, for example sulphur
hexafluoride and perfluorocarbons such as

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perfluoropropane, perfluorobutanes and
perfluoropentanes, may be particularly advantageous in
view of the recognised high stability in the bloodstream
of microbubbles containing such gases.

5　　　　　The gas may comprise a substance such as butane,
cyclobutane, n-pentane, isopentane, neopentane,
cyclopentane, perfluoropentane, perfluorocyclopentane,
perfluorohexane or a mixture containing one or more such
gases which is liquid at handling or processing

10　　　temperatures but gaseous at body temperature, e.g. as
described in the aforementioned WO-A-9416739, since the
film-forming surfactant monolayers in reporter units
according to the invention may stabilise the resulting
microbubbles against uncontrollable growth.

15　　　　　In principle, any appropriate film-forming
surfactant may be employed to form the gas-encapsulating
monolayers, including non-polymeric and non-
polymerisable wall-forming surfactant materials, e.g. as
described in WO-A-9521631; polymer surfactant material,

20　　　e.g. as described in WO-A-9506518; and phospholipids,
e.g. as described in WO-A-9211873, WO-A-9217212, WO-A-
9222247, WO-A-9428780, WO-A-9503835 or WO-A-9729783.
Advantageously 75%, preferably substantially all, of the
film-forming surfactant present in agents according to

25　　　the invention is incorporated into monolayers at the
gas-liquid interfaces.

　　　　　Representative examples of useful phospholipids
include lecithins (i.e. phosphatidylcholines), for
example natural lecithins such as egg yolk lecithin or

30　　　soya bean lecithin and synthetic or semisynthetic
lecithins such as dimyristoylphosphatidylcholine,
dipalmitoylphosphatidylcholine or
distearoylphosphatidylcholine; phosphatidic acids;
phosphatidylethanolamines; phosphatidylserines;

35　　　phosphatidylglycerols; phosphatidylinositols;
cardiolipins; sphingomyelins; fluorinated analogues of
any of the foregoing; mixtures of any of the foregoing

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and mixtures with other lipids such as cholesterol.

It has been found that the use of phospholipids predominantly (e.g. at least 75%) comprising molecules individually bearing net overall charge may be

5   particularly advantageous, especially when used as essentially the sole amphiphilic component of the reporter, and may convey valuable benefits in terms of parameters such as product stability and acoustic properties. Without wishing to be bound by theoretical

10   considerations, it is believed that electrostatic repulsion between charged phospholipid membranes may encourage the formation of stable monolayers at the gas-liquid interfaces; as noted above, the flexibility and deformability of such thin membranes will enhance the

15   echogenicity of reporters used in accordance with the invention relative to gas-filled liposomes comprising one or more lipid bilayers.

The use of charged phospholipids may also provide reporters with advantageous properties regarding, for

20   example, stability, dispersibility and resistance to coalescence without recourse to additives such as further surfactants and/or viscosity enhancers, thereby ensuring that the number of components administered to the body of a subject upon injection of the contrast

25   agents is kept to a minimum. Thus, for example, the charged surfaces of the microbubbles may minimise or prevent their aggregation as a result of electrostatic repulsion.

Desirably at least 75%, preferably substantially

30   all of phospholipid material used in reporters in agents of the invention consists of molecules bearing a net overall charge under conditions of preparation and/or use, which charge may be positive or, more preferably, negative. Representative positively charged

35   phospholipids include esters of phosphatidic acids such as dipalmitoylphosphatidic acid or distearoyl-phosphatidic acid with aminoalcohols such as

hydroxyethylethylenediamine. Examples of negatively charged phospholipids include naturally occurring (e.g. soya bean or egg yolk derived), semisynthetic (e.g. partially or fully hydrogenated) and synthetic phosphatidylserines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids and cardiolipins. The fatty acyl groups of such phospholipids will typically each contain about 14-22 carbon atoms, for example as in palmitoyl and stearoyl groups. Lyso forms of such charged phospholipids are also useful in accordance with the invention, the term "lyso" denoting phospholipids containing only one fatty acyl group, this preferably being ester-linked to the 1-position carbon atom of the glyceryl moiety. Such lyso forms of charged phospholipids may advantageously be used in admixture with charged phospholipids containing two fatty acyl groups.

Phosphatidylserines represent particularly preferred phospholipids of use in agents according to the invention and preferably constitute a substantial part, e.g. at least 80% of the phospholipid content thereof, for example 85-92%. While we do not wish to be bound by theoretical considerations, it may be that ionic bridging between the carboxyl and amino groups of adjacent serine moieties contributes to the stability of such reporter systems. Preferred phosphatidylserines include saturated (e.g. hydrogenated or synthetic) natural phosphatidylserine and synthetic distearoylphosphatidylserine, dipalmitoylphosphatidyl-serine and diarachidoylphosphatidylserine.

Other potentially useful lipids include phosphatidylethanolamines optionally admixed with one or more lipids such as stearic acid, palmitic acid, stearylamine, palmitylamine, cholesterol, bisalkyl glycerols, sphingoglycolipids, synthetic lipids such as N,N-dimethyl-N-octadecyl-1-octadecanammonium chloride or bromide (DODAC, DODAB), and/or maleic acid

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bisalkylesters.

Additional exemplary lipids which may be used to prepare gas-containing contrast agents include fatty acids, stearic acid, palmitic acid, 2-n-hexadecylstearic acid, oleic acid and other acid-containing lipid structures. Such lipid structures may be coupled by amide bond formation to amino acids containing one or more amino groups; the resulting lipid-modified amino acids (e.g. dipalmitoyllysine or distearoyl-2,3-diaminopropionic acid) may be useful precursors for the attachment of functionalised spacer elements having coupling sites for conjugation of one or more vector molecules.

Further useful stabilisers include lipopeptides comprising a lipid attached to a peptide linker portion which is suitably functionalised for coupling to one or more vector molecules. A particular preference is the inclusion of a positively charged peptide linker element (e.g. comprising two or more lysine residues) capable of anchoring through electrostatic interaction with reporter microbubbles stabilised by negatively charged phospholipid or other surfactant membranes.

Another embodiment of the invention comprises functionalised microbubbles carrying one or more reactive groups for non-specific reaction with receptor molecules located on cell surfaces. Microbubbles comprising a thiol moiety, for example, may bind to cell surface receptors via disulphide exchange reactions. The reversible nature of such reactions means that microbubble flow may be controlled by altering the redox environment. Similarly, functionalised microbubbles with membranes comprising activated esters such as N-hydroxysuccinimide esters may be used to react with amino groups found on a multiplicity of cell surface molecules.

Previously proposed microbubble-containing contrast agents based on phospholipids, for example as

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described in WO-A-9409829, are typically prepared by
contacting powdered surfactant, e.g. freeze-dried
preformed liposomes or freeze-dried or spray-dried
phospholipid solutions, with air or other gas and then

5   with aqueous carrier, agitating to generate a
microbubble suspension which must then be administered
shortly after its preparation. Such processes, however,
suffer the disadvantages that substantial agitational
energy must be imparted to generate the required

10   dispersion and that the size and size distribution of
the microbubbles are dependent on the amount of energy
applied and so cannot in practice be controlled.

The reporters or agents according to the present
invention, on the other hand, may advantageously be

15   prepared by generating a gas microbubble dispersion in
an appropriate surfactant (e.g. phospholipid)-containing
aqueous medium, which may if desired previously have
been autoclaved or otherwise sterilised, and then,
preferably after washing and/or size fractionation of

20   the thus-formed microbubbles, subjecting the dispersion
to lyophilisation, e.g. in the presence of one or more
cryoprotectants/lyoprotectants, to yield a dried product
which is readily reconstitutable in water/aqueous
solutions to generate consistently reproducible

25   microbubble dispersions. This process is described in
greater detail in WO-A-9729783, the contents of which
are incorporated herein by reference; the ability to
remove bubbles of unwanted size and excess surfactant
material render this process of substantial advantage

30   over processes such as those described in the
aforementioned WO-A-9409829 and in prior art such as WO-
A-9608234 (where bubbles are generated on site prior to
injection by shaking a suspension of different
phospholipids and viscosity enhancers such as propylene

35   glycol and glycerol).

The above-described process may be used to
generate reporter microbubbles with a very narrow size

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distribution, e.g. such that over 90% (e.g. at least
95%, preferably at least 98%) of the microbubbles have
volume mean diameter in the range 1-7 μm and less than
5% (e.g. not more than 3%, preferably not more than 2%)
of the microbubbles have volume mean diameter above 7
μm. The washing step may be used to ensure that the
reporter is substantially free of unwanted components
such as excess lipids or viscosity enhancers. Agents
containing reporters prepared in this way may exhibit
the following advantages over prior art contrast agent
materials:

        Echogenicity per dose may be greatly enhanced
since substantially all of the surfactant material
participate in stabilisation of the microbubbles as
monolayers. In vivo ultrasound tests in dogs have shown
that ultrasound contrast agents prepared as above may
produce an increase in backscattered signal intensity
from the myocardium of 15 dB following intravenous
injection of doses as low as 0.1 μl microbubbles/kg body
weight.

        Safety in vivo is improved for the same reasons,
since such agents may, for example, be administered in
doses such that the amount of phospholipid injected is
as low as 0.1-10 μg/kg body weight, e.g. 1-5 μg/kg. The
use of such low levels of surfactant may clearly be of
substantial advantage in minimising possible toxic side
effects.

        The high efficacy/dose ratio is also particularly
advantageous in targeting applications, since it is
generally understood that rather low amounts of reporter
will accumulate at sites of interest when using products
comprising vectors having affinity for such sites.
These preferred reporters according to the invention may
therefore considerably improve contrast at sites of
interest compared to known targetable ultrasound
contrast agents. Their high efficacy may effectively
make it possible to "see" single microbubbles using

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ultrasound, giving a sensitivity close to or potentially
even higher than that of scintigraphy, which currently
is probably the most useful technique in targeting,
although the resolution in scintigraphic pictures is not
impressive.

A particular advantage of phosphatidylserine-based
agents is their biocompatibility; thus no acute toxic
effects such as changes in blood pressure or heart rate
have been observed in animal tests on dogs injected with
intravenous boluses of phosphatidylserine-based contrast
agents prepared as described above at doses of up to ten
times a normal imaging dose.

The use of charged phospholipids may also be of
advantage in that they will contain functional groups
such as carboxyl or amino which permit ready linking of
vectors, if desired by way of linking units. It should
be noted that other functional groups may also be
incorporated into such systems by mixing a lipid
containing a desired functional group with the film-
forming surfactant prior to microbubble generation.

It is generally unnecessary to incorporate
additives such as emulsifying agents and/or viscosity
enhancers such as are commonly employed in many existing
contrast agent formulations into agents of the
invention. As noted above, this is of advantage in
keeping to a minimum the number of components
administered to the body of a subject and ensuring that
the viscosity of the agents is as low as possible.
Since preparation of the agents typically involves a
freeze drying step as discussed above, it may however be
advantageous to include a cryoprotectant/lyoprotectant
or bulking agent, for example an alcohol, e.g. an
aliphatic alcohol such as t-butanol; a polyol such as
glycerol; a carbohydrate, e.g. a sugar such as sucrose,
mannitol, trehalose or a cyclodextrin, or a
polysaccharide such as dextran; or a polyglycol such as
polyethylene glycol. The use of physiologically well-

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tolerated sugars such as sucrose is preferred.

Lyophilised dried products prepared as described above are especially readily reconstitutable in water, requiring only minimal agitation such as may, for example, be provided by gentle hand-shaking for a few seconds. The size of the microbubbles so generated is consistently reproducible and is independent of the amount of agitational energy applied, in practice being determined by the size of the microbubbles formed in the initial microbubble dispersion; surprisingly this size parameter is substantially maintained in the lyophilised and reconstituted product. Thus, since the size of the microbubbles in the initial dispersion may readily be controlled by process parameters such as the method, speed and duration of agitation, the final microbubble size may readily be controlled.

The lyophilised dried products have also proved to be storage stable for at least several months under ambient conditions. The microbubble dispersions generated upon reconstitution in water are stable for at least 8 hours, permitting considerable flexibility as to when the dried product is reconstituted prior to injection.

The high efficacy of these preferred reporters may make it possible to use smaller bubbles than usual while still generating ultrasound contrast effects significantly above the minimum detection levels of current ultrasound imaging equipment. Such smaller bubbles have potential advantages such as reduced clogging of vessels, longer circulation times, greater ability to reach targets, and lower accumulation in lungs or other non-target organs, and their use and agents containing them constitute further features of the invention.

It may also be possible to use such smaller bubbles to exploit the enhanced ultrasound contrast effects of bubble clusters. It is known from theory

that the ultrasound contrast effect of a specific number
of bubbles with total volume V in a dilute dispersion
increases when the bubbles aggregate to form a larger
gas phase with the same total volume V. It may

5   therefore be possible to use small bubbles which give
substantially no ultrasound contrast until they are
clustered (as may occur in target areas in preference to
non-target sites having low densities of target
molecules). Small bubbles may also be designed to fuse,

10   e.g. through interbubble binding promoted by interaction
with the target, so as to enhance contrast in target
areas. Interbubble crosslinking and consequent
clustering may also be effected if the reporter, in
addition to carrying a vector leading to retention at

15   specific sites, has unreacted linker moieties capable of
reaction with functional groups on other bubbles.

        Within the context of the present invention, the
reporter unit will usually remain attached to the
vectors. However, in one type of targeting procedure,

20   sometimes called "pre-targeting", the vector (often a
monoclonal antibody) is administered alone; subsequently
the reporter is administered, coupled to a moiety which
is capable of specifically binding the pre-targeting
vector molecule (when the pre-targeting vector is an

25   antibody, the reporter may be coupled to an
immunoglobulin-binding molecule, such as protein A or an
anti-immunoglobulin antibody). The advantage of this
protocol is that time may be allowed for elimination of
the vector molecules that do not bind their targets,

30   substantially reducing the background problems that are
connected with the presence of an excess of reporter-
vector conjugate. Within the context of the present
invention, pre-targeting with one specific vector might
be envisaged, followed by reporter units that are

35   coupled to another vector and a moiety which binds the
first vector.

        Again in the context of the present invention, for

example in assessment of blood perfusion rates in
targeted areas such as the myocardium, it is of interest
to measure the rate at which contrast agents bound to
the target are displaced or released therefrom. This
5   may be achieved in a controlled manner by administration
of an additional vector and/or other substance able to
displace or release the contrast agent from its target.

Ultrasound imaging modalities which may be used in
accordance with the invention include two- and three-
10   dimensional imaging techniques such as B-mode imaging
(for example using the time-varying amplitude of the
signal envelope generated from the fundamental frequency
of the emitted ultrasound pulse, from sub-harmonics or
higher harmonics thereof or from sum or difference
15   frequencies derived from the emitted pulse and such
harmonics, images generated from the fundamental
frequency or the second harmonic thereof being
preferred), colour Doppler imaging and Doppler amplitude
imaging, and combinations of the two latter with any of
20   the above modalities. Surprisingly excellent second
harmonic signals have been obtained from targeted
monolayer-stabilised microspheres in accordance with the
present invention. To reduce the effects of movement,
successive images of tissues such as the heart or kidney
25   may be collected with the aid of suitable
synchronisation techniques (e.g. gating to the ECG or
respiratory movement of the subject). Measurement of
changes in resonance frequency or frequency absorption
which accompany arrested or retarded microbubbles may
30   also usefully be made to detect the contrast agent.

The present invention provides a tool for
therapeutic drug delivery in combination with vector-
mediated direction of the product to the desired site.
By "therapeutic" or "drug" is meant an agent having a
35   beneficial effect on a specific disease in a living
human or non-human animal. Whilst combinations of drugs
and ultrasound contrast agents have been proposed in,

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for example, WO-A-9428873 and WO-A-9507072, these
products lack vectors having affinity for particular
sites and thereby show comparitively poor specific
retention at desired sites prior to or during drug
5   release.

        Therapeutic compounds used in accordance with the
present invention may be encapsulated in the interior of
the microbubbles or attached to or incorporated in the
stabilising membranes. Thus, the therapeutic compound
10   may be linked to a part of the membrane, for example
through covalent or ionic bonds, or may be physically
mixed into the stabilising material, particularly if the
drug has similar polarity or solubility to the membrane
material, so as to prevent it from leaking out of the
15   product before it is intended to act in the body. The
release of the drug may be initiated merely by wetting
contact with blood following administration or as a
consequence of other internal or external influences,
e.g. dissolution processes catalyzed by enzymes or the
20   use of of ultrasound. The destruction of gas-containing
microparticles using external ultrasound is a well known
phenomenon in respect of ultrasound contrast agents,
e.g. as described in WO-A-9325241; the rate of drug
release may be varied depending on the type of
25   therapeutic application, using a specific amount of
ultrasound energy from the transducer.

        The therapeutic may be covalently linked to the
encapsulating membrane surface using a suitable linking
agent, e.g. as described herein. Thus, for example, one
30   may initially prepare a phospholipid or lipopeptide
derivative to which the drug is bonded through a
biodegradable bond or linker, and then incorporate this
derivative into the material used to prepare the
reporter, as described above.

35   Representative therapeutics suitable for use in
the present drug delivery compositions include any known
therapeutic drugs or active analogues thereof containing

thiol groups which may be coupled to thiol-containing
microbubbles under oxidative conditions yielding
disulphide groups. In combination with a vector or
vectors such drug/vector-modified microbubbles may be
allowed to accumulate in target tissue; administration
of a reducing agent such as reduced glutathione may then
liberate the drug molecule from the targeted microbubble
in the vicinity of the target cell, increasing the local
concentration of the drug and enhancing its therapeutic
effect. Alternatively the composition may initially be
prepared without the therapeutic, which may then be
coupled to or coated on the microbubbles immediately
prior to use; thus, for example, a therapeutic may be
added to a suspension of microbubbles in aqueous media
and shaken in order to attach or adhere the therapeutic
to the microbubbles.

Other drug delivery systems include vector-
modified phospholipid membranes doped with lipopeptide
structures comprising a poly-L-lysine or poly-D-lysine
chain in combination with a targeting vector. Applied
to gene therapy/antisense technologies with particular
emphasis on receptor-mediated drug delivery, the
microbubble carrier is condensed with DNA or RNA via
elecrostatic interaction with the cationic polylysine.
This method has the advantage that the vector or vectors
used for targeted delivery are not directly attached to
the polylysine carrier moiety. The polylysine chain is
also anchored more tightly in the microbubble membrane
due to the presence of the lipid chains. The use of
ultrasound to increase the effectiveness of delivery is
also considered useful.

Alternatively free polylysine chains are firstly
modified with drug or vector molecules then condensed
onto the negative surface of targeted microbubbles.

Representative and non-limiting examples of drugs
useful in accordance with the invention include
antineoplastic agents such as vincristine, vinblastine,

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vindesine, busulfan, chlorambucil, spiroplatin,
cisplatin, carboplatin, methotrexate, adriamycin,
mitomycin, bleomycin, cytosine arabinoside, arabinosyl
adenine, mercaptopurine, mitotane, procarbazine,

5   dactinomycin (antinomycin D), daunorubicin, doxorubicin
hydrochloride, taxol, plicamycin, aminoglutethimide,
estramustine, flutamide, leuprolide, megestrol acetate,
tamoxifen, testolactone, trilostane, amsacrine (m-AMSA),
asparaginase (L-asparaginase), etoposide, interferon a-

10   2a and 2b, blood products such as hematoporphyrins or
derivatives of the foregoing; biological response
modifiers such as muramylpeptides; antifungal agents
such as ketoconazole, nystatin, griseofulvin,
flucytosine, miconazole or amphotericin B; hormones or

15   hormone analogues such as growth hormone, melanocyte
stimulating hormone, estradiol, beclomethasone
dipropionate, betamethasone, cortisone acetate,
dexamethasone, flunisolide, hydrocortisone,
methylprednisolone, paramethasone acetate, prednisolone,

20   prednisone, triamcinolone or fludrocortisone acetate;
vitamins such as cyanocobalamin or retinoids; enzymes
such as alkaline phosphatase or manganese superoxide
dismutase; antiallergic agents such as amelexanox;
inhibitors of tissue factor such as monoclonal

25   antibodies and Fab fragments thereof, synthetic
peptides, nonpeptides and compounds downregulating
tissue factor expression; inhibitors of platelets such
as GPIa, GPIb and GPIIb-IIIa, ADP receptors, thrombin
receptors, von Willebrand factor, prostaglandins,

30   aspirin, ticlopidin, clopigogrel and reopro; inhibitors
of coagulation protein targets such as FIIa, FVa, FVIIa,
FVIIIA, FIXa, FXa, tissue factor, heparins, hirudin,
hirulog, argatroban, DEGR-rFVIIa and annexin V:
inhibitors of fibrin formation and promoters of

35   fibrinolysis such as t-PA, urokinase, Plasmin,
Streptokinase, rt-Plasminogen Activator and
rStaphylokinase; antiangiogenic factors such as

medroxyprogesteron, pentosan polysulphate, suramin,
taxol, thalidomide, angiostatin, interferon-alpha,
metalloproteinase inhibitors, platelet factor 4,
somatostatin, thromobospondin; circulatory drugs such as
propranolol; metabolic potentiators such as glutathione;
antituberculars such as p-aminosalicylic acid,
isoniazid, capreomycin sulfate, cyclosexine, ethambutol,
ethionamide, pyrazinamide, rifampin or streptomycin
sulphate; antivirals such as acyclovir, amantadine,
azidothymidine, ribavirin or vidarabine; blood vessel
dilating agents such as diltiazem, nifedipine,
verapamil, erythritol tetranitrate, isosorbide
dinitrate, nitroglycerin or pentaerythritol
tetranitrate; antibiotics such as dapsone,
chloramphenicol, neomycin, cefaclor, cefadroxil,
cephalexin, cephradine, erythromycin, clindamycin,
lincomycin, amoxicillin, ampicillin, bacampicillin,
carbenicillin, dicloxacillin, cyclacillin,
picloxacillin, hetacillin, methicillin, nafcillin,
penicillin, polymyxin or tetracycline;
antiinflammatories such as diflunisal, ibuprofen,
indomethacin, meclefenamate, mefenamic acid, naproxen,
phenylbutazone, piroxicam, tolmetin, aspirin or
salicylates; antiprotozoans such as chloroquine,
metronidazole, quinine or meglumine antimonate;
antirheumatics such as penicillamine; narcotics such as
paregoric; opiates such as codeine, morphine or opium;
cardiac glycosides such as deslaneside, digitoxin,
digoxin, digitalin or digitalis; neuromuscular blockers
such as atracurium mesylate, gallamine triethiodide,
hexafluorenium bromide, metocurine iodide, pancuronium
bromide, succinylcholine chloride, tubocurarine chloride
or vecuronium bromide; sedatives such as amobarbital,
amobarbital sodium, apropbarbital, butabarbital sodium,
chloral hydrate, ethchlorvynol, ethinamate, flurazepam
hydrochloride, glutethimide, methotrimeprazine
hydrochloride, methyprylon, midazolam hydrochloride,

paraldehyde, pentobarbital, secobarbital sodium,
talbutal, temazepam or triazolam; local anaesthetics
such as bupivacaine, chloroprocaine, etidocaine,
lidocaine, mepivacaine, procaine or tetracaine; general
anaesthetics such as droperidol, etomidate, fentanyl
citrate with droperidol, ketamine hydrochloride,
methohexital sodium or thiopental and pharmaceutically
acceptable salts (e.g. acid addition salts such as the
hydrochloride or hydrobromide or base salts such as
sodium, calcium or magnesium salts) or derivatives (e.g.
acetates) thereof. Other examples of therapeutics
include genetic material such as nucleic acids, RNA, and
DNA of natural or synthetic origin, including
recombinant RNA and DNA. DNA encoding certain proteins
may be used in the treatment of many different types of
diseases. For example, tumor necrosis factor or
interleukin-2 genes may be provided to treat advanced
cancers; thymidine kinase genes may be provided to
treat ovarian cancer or brain tumors; interleukin-2
genes may be provided to treat neuroblastoma, malignant
melanoma or kidney cancer; and interleukin-4 genes may
be provided to treat cancer.

        Lipophilic derivatives of drugs linked to the
microbubble membrane through hydrophobic interactions
may exhibit therapeutic effects as part of the
microbubble or after release from the microbubble, e.g.
by use of ultrasound. If the drug does not possess the
desired physical properties, a lipophilic group may be
introduced for anchoring the drug to the membrane.
Preferably the lipophilic group should be introduced in
a way that does not influence the *in vivo* potency of the
molecule, or the lipophilic group may be cleaved
releasing the active drug. Lipophilic groups may be
introduced by various chemical means depending on
functional groups available in the drug molecule.
Covalent coupling may be effected using functional
groups in the drug molecule capable of reacting with

appropriately functionalised lipophilic compounds. Examples of lipophilic moieties include branched and unbranched alkyl chains, cyclic compounds, aromatic residues and fused aromatic and non-aromatic cyclic systems. In some instances the lipophilic moiety will consist of a suitably functionalised steroid, such as cholesterol or a related compound. Examples of functional groups particularly suitable for derivatisation include nucleophilic groups like amino, hydroxy and sulfhydryl groups. Suitable processes for lipophilic derivatisation of any drug containing a sulfhydryl group, such as captopril, may include direct alkylation, e.g. reaction with an alkyl halide under basic conditions and thiol ester formation by reaction with an activated carboxylic acid. Representative examples of derivatisation of any drug having carboxylic functions, for example atenolol or chlorambucil, include amide and ester formation by coupling respectively with amines and alcohols possessing appropriate physical properties. A preferred embodiment comprises attachment of cholesterol to a therapeutic compound by forming a degradable ester bond.

A preferred application of the present invention relates to *angiogenesis*, which is the formation of new blood vessels by branching from existing vessels. The primary stimulus for this process may be inadequate supply of nutrients and oxygen (hypoxia) to cells in a tissue. The cells may respond by secreting angiogenetic factors, of which there are many; one example is *vascular endothelial growth factor*. These factors initiate the secretion of proteolytic enzymes which break down the proteins of the basement membrane, as well as inhibitors which limit the action of these potentially harmful enzymes. The combined effect of loss of attachment and signals from the receptors for angiogenetic factors is to cause the endothelial cells to move, multiply, and rearrange themselves, and finally

to synthesise a basement membrane around the new
vessels.

Tumors must initiate angiogenesis when they reach
millimeter size in order to keep up their rate of
growth. As angiogenesis is accompanied by
characteristic changes in the endothelial cells and
their environment, this process is a promising target
for therapeutic intervention. The transformations
accompanying angiogenesis are also very promising for
diagnosis, a preferred example being malignant disease,
but the concept also shows great promise in inflammation
and a variety of inflammation-related diseases. These
factors are also involved in re-vascularisation of
infarcted parts of the myocardium, which occurs if a
stenosis is released within a short time.

A number of known receptors/targets associated
with angiogenesis are given in subsequent tables. Using
the targeting principles described in the present
disclosure, angiogenesis mav be detected by the majority
of the imaging modalities in use in medicine.
Contrast-enhanced ultrasound may possess additional
advantages, the contrast medium being microspheres which
are restricted to the interior of blood vessels. Even
if the target antigens are found on many cell types, the
microspheres will attach exclusively to endothelial
cells.

So-called prodrugs may also be used in agents
according to the invention. Thus drugs may be
derivatised to alter their physicochemical properties
and to adapt them for inclusion into the reporter; such
derivatised drugs may be regarded as prodrugs and are
usually inactive until cleavage of the derivatising
group regenerates the active form of the drug.

By targeting gas-filled microbubbles containing a
prodrug-activating enzyme to areas of pathology, one may
image targeting the enzyme, making it possible to
visualise when the microbubbles are targeted properly to

the area of pathology and at the same time have
disappeared from non-target areas. In this way one can
determine the optimal time for injection of prodrug
into individual patients.

5   Another alternative is to incorporate the prodrug,
prodrug-activating enzyme and vector in the same
microbubbles in a system where the prodrug will only be
activated after some external stimulus. Such a stimulus
may, for example, be a tumour-specific protease as
10   described above, or bursting of the microbubbles by
external ultrasound after the desired targeting has been
achieved.

Therapeutics may easily be delivered in accordance
with the invention to diseased or necrotic areas, for
15   example in the heart, general vasculature, and to the
liver, spleen, kidneys and other regions such as the
lymph system, body cavities or gastrointestinal system.

Products according to the present invention may be
used for targeted therapeutic delivery either *in vivo* or
20   *in vitro*. In the latter context the products may be
useful in *in vitro* systems such as kits for diagnosis of
different diseases or characterisation of different
components in blood or tissue samples. Similar
techniques to those used to attach certain blood
25   components or cells to polymer particles (e.g.
monodisperse magnetic particles) *in vitro* to separate
them from a sample may be used in the present invention,
using the low density of the reporter units in agents of
the present invention to effect separation of the gas-
30   containing material by flotation and repeated washing.

Coupling of a reporter unit to a desired vector
(and/or therapeutic drug) may be achieved by covalent or
non-covalent means, usually involving interaction with
one or more functional groups located on the reporter
35   and/or vector and/or any intervening linker group/spacer
element. Examples of chemically reactive functional
groups which may be employed for this purpose include

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amino, hydroxyl, sulfhydryl, carboxyl, and carbonyl
groups, as well as carbohydrate groups, vicinal diols,
thioethers, 2-aminoalcohols, 2-aminothiols, guanidinyl,
imidazolyl and phenolic groups.

5   Covalent coupling of reporter and vector may
therefore be effected using linking agents containing
reactive moities capable of reaction with such
functional groups. Examples of reactive moieties
capable of reaction with sulfhydryl groups include α-

10   haloacetyl compounds of the type X-CH₂CO- (where X=Br, Cl
or I), which show particular reactivity for sulfhydryl
groups but which can also be used to modify imidazolyl,
thioether, phenol and amino groups as described by Gurd,
F.R.N. in *Methods Enzymol.* (1967) **11**, 532. N-Maleimide

15   derivatives are also considered selective towards
sulfhydryl groups, but may additionaly be useful in
coupling to amino groups under certain conditions. N-
maleimides may be incorporated into linking systems for
reporter-vector conjugation as described by Kitagawa, T.

20   *et al.* in *Chem. Pharm. Bull.* (1981) **29**, 1130 or used as
polymer crosslinkers for bubble stabilisation as
described by Kovacic, P. *et al.* in *J. Am. Chem. Soc.*
(1959) **81**, 1887. Reagents such as 2-iminothiolane, e.g.
as described by Traut, R. *et al.* in *Biochemistry* (1973)

25   **12**, 3266, which introduce a thiol group through
conversion of an amino group, may be considered as
sulfhydryl reagents if linking occurs through the
formation of disulphide bridges. Thus reagents which
introduce reactive disulphide bonds into either the

30   reporter or the vector may be useful, since linking may
be brought about by disulphide exchange between the
vector and reporter; examples of such reagents include
Ellman's reagent (DTNB), 4,4'-dithiodipyridine, methyl-
3-nitro-2-pyridyl disulphide and methyl-2-pyridyl

35   disulphide (described by Kimura, T. *et al.* in *Analyt.
Biochem.* (1982) **122**, 271).

        Examples of reactive moieties capable of reaction

with amino groups include alkylating and acylating agents. Representative alkylating agents include:

i) α-haloacetyl compounds, which show specificity towards amino groups in the absence of reactive thiol groups and are of the type X-CH$_2$CO- (where X=Cl, Br or I), e.g. as described by Wong, Y-H.H. in *Biochemistry* (1979) **24**, 5337;

ii) N-maleimide derivatives, which may react with amino groups either through a Michael type reaction or through acylation by addition to the ring carbonyl group as described by Smyth, D.G. et al. in *J. Am. Chem. Soc.* (1960) **82**, 4600 and *Biochem. J.* (1964) **91**, 589;

iii) aryl halides such as reactive nitrohaloaromatic compounds;

iv) alkyl halides as described by McKenzie, J.A. et al. in *J. Protein Chem.* (1988) **7**, 581;

v) aldehydes and ketones capable of Schiff's base formation with amino groups, the adducts formed usually being stabilised through reduction to give a stable amine;

vi) epoxide derivatives such as epichlorohydrin and bisoxiranes, which may react with amino, sulfhydryl or phenolic hydroxyl groups;

vii) chlorine-containing derivatives of s-triazines, which are very reactive towards nucleophiles such as amino, sufhydryl and hydroxy groups;

viii) aziridines based on s-triazine compounds detailed above, e.g. as described by Ross, W.C.J. in *Adv. Cancer Res.* (1954) **2**, 1, which react with nucleophiles such as amino groups by ring opening;

ix) squaric acid diethyl esters as described by Tietze, L.F. in *Chem. Ber.* (1991) **124**, 1215; and

x) α-haloalkyl ethers, which are more reactive alkylating agents than normal alkyl halides because of the activation caused by the ether oxygen atom, e.g. as described by Benneche, T. et al. in *Eur. J. Med. Chem.* (1993) **28**, 463.

Representative amino-reactive acylating agents include:

i)   isocyanates and isothiocyanates, particularly aromatic derivatives, Which form stable urea and thiourea derivatives respectively and have been used for protein crosslinking as described by Schick, A.F. et al. in J. Biol. Chem. (1961) **236**, 2477;

ii)   sulfonyl chlorides, which have been described by Herzig, D.J. et al. in Biopolymers (1964) **2**, 349 and which may be useful for the introduction of a fluorescent reporter group into the linker;

iii)   Acid halides;

iv)   Active esters such as nitrophenylesters or N-hydroxysuccinimidyl esters;

v)   acid anhydrides such as mixed, symmetrical or N-carboxyanhydrides;

vi)   other useful reagents for amide bond formation as described by Bodansky, M. et al. in `Principles of Peptide Synthesis' (1984) Springer-Verlag;

vii)   acylazides, e.g. wherein the azide group is generated from a preformed hydrazide derivative using sodium nitrite, e.g. as described by Wetz, K. et al. in Anal. Biochem. (1974) **58**, 347;

viii) azlactones attached to polymers such as bis-acrylamide, e.g. as described by Rasmussen, J.K. in Reactive Polymers (1991) **16**, 199; and

ix)   Imidoesters , which form stable amidines on reaction with amino groups, e.g. as described by Hunter, M.J. and Ludwig, M.L. in J. Am. Chem. Soc. (1962) **84**, 3491.

Carbonyl groups such as aldehyde functions may be reacted with weak protein bases at a pH such that nucleophilic protein side-chain functions are protonated. Weak bases include 1,2-aminothiols such as those found in N-terminal cysteine residues, which selectively form stable 5-membered thiazolidine rings with aldehyde groups, e.g. as described by Ratner, S. et

al. in *J. Am. Chem. Soc.* (1937) **59**, 200. Other weak bases such as phenyl hydrazones may be used, e.g. as described by Heitzman, H. *et al.* in *Proc. Natl. Acad. Sci. USA* (1974) **71**, 3537.

Aldehydes and ketones may also be reacted with amines to form Schiff's bases, which may advantageously be stabilised through reductive amination. Alkoxylamino moieties readily react with ketones and aldehydes to produce stable alkoxamines, e.g. as described by Webb, R. *et al.* in *Bioconjugate Chem.* (1990) **1**, 96.

Examples of reactive moieties capable of reaction with carboxyl groups include diazo compounds such as diazoacetate esters and diazoacetamides, which react with high specificity to generate ester groups, e.g. as described by Herriot R.M. in *Adv. Protein Chem.* (1947) **3**, 169. Carboxylic acid modifying reagents such as carbodiimides, which react through O-acylurea formation followed by amide bond formation, may also usefully be employed; linking may be facilitated through addition of an amine or may result in direct vector-receptor coupling. Useful water soluble carbodiimides include 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide (CMC) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), e.g. as described by Zot, H.G. and Puett, D. in *J. Biol. Chem.* (1989) **264**, 15552. Other useful carboxylic acid modifying reagents include isoxazolium derivatives such as Woodwards reagent K; chloroformates such as p-nitrophenylchloroformate; carbonyldiimidazoles such as 1,1'-carbonyldiimidazole; and N-carbalkoxydihydroquinolines such as N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline.

Other potentially useful reactive moieties include vicinal diones such as p-phenylenediglyoxal, which may be used to react with guanidinyl groups, e.g. as described by Wagner *et al.* in *Nucleic acid Res.* (1978) **5**, 4065; and diazonium salts, which may undergo

electrophilic substitution reactions, e.g. as described
by Ishizaka, K. and Ishizaka T. in *J. Immunol.* (1960)
85, 163. Bis-diazonium compounds are readily prepared
by treatment of aryl diamines with sodium nitrite in
acidic solutions. It will be appreciated that functional
groups in the reporter and/or vector may if desired be
converted to other functional groups prior to reaction,
e.g. to confer additional reactivity or selectivity.
Examples of methods useful for this purpose include
conversion of amines to carboxylic acids using reagents
such as dicarboxylic anhydrides; conversion of amines to
thiols using reagents such as N-acetylhomocysteine
thiolactone, S-acetylmercaptosuccinic anhydride, 2-
iminothiolane or thiol-containing succinimidyl
derivatives; conversion of thiols to carboxylic acids
using reagents such as α-haloacetates; conversion of
thiols to amines using reagents such as ethylenimine or
2-bromoethylamine; conversion of carboxylic acids to
amines using reagents such as carbodiimides followed by
diamines; and conversion of alcohols to thiols using
reagents such as tosyl chloride followed by
transesterification with thioacetate and hydrolysis to
the thiol with sodium acetate.

Vector-reporter coupling may also be effected
using enzymes as zero-length linking agents; thus, for
example, transglutaminase, peroxidase and xanthine
oxidase may be used to produce linked products. Reverse
proteolysis may also be used for linking through amide
bond formation.

Non-covalent vector-reporter coupling may, for
example, be effected by electrostatic charge
interactions e.g. between a polylysinyl-functionalised
reporter and a polyglutamyl-functionalised vector,
through chelation in the form of stable metal complexes
or through high affinity binding interaction such as
avidin/biotin binding. Polylysine, coated non-
covalently to a negatively charged membrane surface may

also increase non-specifically the affinity of a
microbubble for a cell through charge interactions.

Alternatively, a vector may be coupled to a
protein known to bind phospholipids. In many instances,
a single molecule of phospholipid may attach to a
protein such as a translocase, while other proteins may
attach to surfaces consisting mainly of phospholipid
head groups and so may be used to attach vectors to
phospholipid microspheres; one example of such a protein
is ß2-glycoprotein I (Chonn, A., Semple, S.C. and
Cullis, P.R., *Journal of Biological Chemistry* (1995)
270, 25845-25849). Phosphatidylserine-binding proteins
have been described, e.g. by Igarashi, K. *et al.* in
Journal of Biological Chemistry **270(49)**, 29075-29078; a
conjugate of a vector with such a phosphatidylserine-
binding protein may therefore be used to attach the
vector to phosphatidylserine-encapsulated microbubbles.
When the amino acid sequence of a binding protein is
known, the phospholipid-binding portion may be
synthesised or isolated and used for conjugation with a
vector, thus avoiding the biological activity which may
be located elsewhere in the molecule.

It is also possible to obtain molecules that bind
specifically to the surface (or in the "membrane") of
microspheres by direct screening of molecular libraries
for microsphere-binding molecules. For example, phage
libraries displaying small peptides may be used for such
selection. The selection may be made by simply mixing
the microspheres and the phage display library and
eluting the phages binding to the floating microspheres.
If desired, the selection may be done under
"physiological conditions" (e.g. in blood) to eliminate
peptides which cross-react with blood components. An
advantage of this type of selection procedure is that
only binding molecules that do not destabilise the
microspheres should be selected, since only binding
molecules attached to intact floating microspheres will

rise to the top. It may also be possible to introduce
some kind of "stress" during the selection procedure
(e.g. pressure) to ensure that destabilising binding
moieties are not selected. Furthermore the selection
may be done under shear conditions, for example by first
letting the phages react with the microspheres and then
letting the microspheres pass through a surface coated
with anti-phage antibodies under flow conditions. In
this way it may be possible to select binders which may
resist shear conditions present *in vivo*. Binding
moieties identified in this way may be coupled (by
chemical conjugation or via peptide synthesis, or at the
DNA-level for recombinant vectors) to a vector molecule,
constituting a general tool for attaching any vector
molecule to the microspheres.

A vector which comprises or is coupled to a
peptide, lipo-oligosaccharide or lipopeptide linker
which contains a element capable of mediating membrane
insertion may also be useful. One example is described
by Leenhouts, J.M. et al. in *Febs Letters* (1995) **370(3)**,
189-192. Non-bioactive molecules consisting of known
membrane insertion anchor/signal groups may also be used
as vectors for certain applications, an example being
the H1 hydrophobic segment from the Na,K-ATPase α-
subunit described by Xie, Y. and Morimoto, T. in *J.
Biol. Chem.* (1995) **270(20)**, 11985-11991. The anchor
group may also be fatty acid(s) or cholesterol.

Coupling may also be effected using avidin or
streptavidin, which have four high affinity binding
sites for biotin. Avidin may therefore be used to
conjugate vector to reporter if both vector and reporter
are biotinylated. Examples are described by Bayer, E.A.
and Wilchek, M. in *Methods Biochem. Anal.* (1980) **26**, 1.
This method may also be extended to include linking of
reporter to reporter, a process which may encourage
bubble association and consequent potentially increased
echogenicity. Alternatively, avidin or streptavidin may

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be attached directly to the surface of reporter
microparticles.

Non-covalent coupling may also utilise the
bifunctional nature of bispecific immunoglobulins.
These molecules can specifically bind two antigens, thus
linking them. For example, either bispecific IgG or
chemically engineered bispecific F(ab)'2 fragments may be
used as linking agents. Heterobifunctional bispecific
antibodies have also been reported for linking two
different antigens, e.g. as described by Bode, C. et al.
in J. Biol. Chem. (1989) 264, 944 and by Staerz, U.D. et
al. in Proc. Natl. Acad. Sci. USA (1986) 83, 1453.
Similarly, any reporter and/or vector containing two or
more antigenic determinants (e.g. as described by Chen,
Aa et al. in Am. J. Pathol.(1988) 130, 216) may be
crosslinked by antibody molecules and lead to formation
of multi-bubble cross-linked assemblies of potentially
increased echogenicity.

Linking agents used in accordance with the
invention will in general bring about linking of vector
to reporter or reporter to reporter with some degree of
specificity, and may also be used to attach one or more
therapeutically active agents.

In some instances it is considered advantageous to
include a PEG component as a stabiliser in conjunction
with a vector or vectors or directly to the reporter in
the same molecule where the PEG does not serve as a
spacer.

So-called zero-length linking agents, which induce
direct covalent joining of two reactive chemical groups
without introducing additional linking material (e.g. as
in amide bond formation induced using carbodiimides or
enzymatically) may, if desired, be used in accordance
with the invention, as may agents such as biotin/avidin
systems which induce non-covalent reporter-vector
linking and agents which induce hydrophobic or
electrostatic interactions.

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Most commonly, however, the linking agent will comprise two or more reactive moieties, e.g. as described above, connected by a spacer element. The presence of such a spacer permits bifunctional linkers to react with specific functional groups within a molecule or between two different molecules, resulting in a bond between these two components and introducing extrinsic linker-derived material into the reporter-vector conjugate. The reactive moieties in a linking agent may be the same (homobifunctional agents) or different (heterobifunctional agents or, where several dissimilar reactive moieties are present, heteromultifunctional agents), providing a diversity of potential reagents that may bring about covalent bonding between any chemical species, either intramolecularly or intermolecularly.

The nature of extrinsic material introduced by the linking agent may have a critical bearing on the targeting ability and general stability of the ultimate product. Thus it may be desirable to introduce labile linkages, e.g. containing spacer arms which are biodegradable or chemically sensitive or which incorporate enzymatic cleavage sites. Alternatively the spacer may include polymeric components, e.g. to act as surfactants and enhance bubble stability. The spacer may also contain reactive moieties, e.g. as described above to enhance surface crosslinking, or it may contain a tracer element such as a fluorescent probe, spin label or radioactive material.

Contrast agents according to the present invention are therefore useful in all imaging modalities since contrast elements such as X-ray contrast agents, light imaging probes, spin labels or radioactive units may readily be incorporated in or attached to the reporter units.

Spacer elements may typically consist of aliphatic chains which effectively separate the reactive moieties

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of the linker by distances of between 5 and 30 Å. They
may also comprise macromolecular structures such as
PEGs, which have been given much attention in
biotechnical and biomedical applications (see e.g.
5   Milton Harris, J. (ed) "*Poly(ethylene glycol) chemistry,
biotechnical and biomedical applications*" Plenum Press,
New York, 1992). PEGs are soluble in most solvents,
including water, and are highly hydrated in aqueous
environments, with two or three water molecules bound to
10   each ethylene glycol segment; this has the effect of
preventing adsorption either of other polymers or of
proteins onto PEG-modified surfaces. PEGs are known to
be nontoxic and not to harm active proteins or cells,
whilst covalently linked PEGs are known to be non-
15   immunogenic and non-antigenic. Furthermore, PEGs may
readily be modified and bound to other molecules with
only little effect on their chemistry. Their
advantageous solubility and biological properties are
apparent from the many possible uses of PEGs and
20   copolymers thereof, including block copolymers such as
PEG-polyurethanes and PEG-polypropylenes.

Appropriate molecular weights for PEG spacers used
in accordance with the invention may, for example, be
between 120 Daltons and 20 kDaltons.

25   The major mechanism for uptake of particles by the
cells of the reticuloendothelial system (RES) is
opsonisation by plasma proteins in blood; these mark
foreign particles which are then taken up by the RES.
The biological properties of PEG spacer elements used in
30   accordance with the invention may serve to increase
contrast agent circulation time in a similar manner to
that observed for PEGylated liposomes (see e.g.
Klibanov, A.L. *et al.* in *FEBS Letters* (1990) **268**, 235-
237 and Blume, G. and Cevc, G. in *Biochim. Biophys. Acta*
35   (1990) **1029**, 91-97). Increased coupling efficiency to
areas of interest may also be achieved using antibodies
bound to the terminii of PEG spacers (see e.g. Maruyama,

K. *et al*. in *Biochim. Biophys. Acta* (1995) **1234**, 74-80
and Hansen, C.B. *et al*. in *Biochim. Biophys. Acta* (1995)
1239, 133-144).

In some instances it is considered advantageous to
include a PEG component as a stabiliser in conjunction
with a vector or vectors or directly to the reporter in
the same molecule where the PEG does not serve as a
spacer.

Other representative spacer elements include
structural-type polysaccharides such as polygalacturonic
acid, glycosaminoglycans, heparinoids, cellulose and
marine polysaccharides such as alginates, chitosans and
carrageenans; storage-type polysaccharides such as
starch, glycogen, dextran and aminodextrans; polyamino
acids and methyl and ethyl esters thereof, as in homo-
and co-polymers of lysine, glutamic acid and aspartic
acid; and polypeptides, oligosaccharides and
oligonucleotides, which may or may not contain enzyme
cleavage sites.

In general, spacer elements may contain cleavable
groups such as vicinal glycol, azo, sulfone, ester,
thioester or disulphide groups. Spacers containing
biodegradable methylene diester or diamide groups of
formula

$$-(Z)_m.Y.X.C(R^1R^2).X.Y.(Z)_n-$$
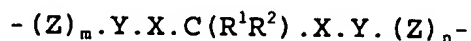
[where X and Z are selected from -O-, -S-, and -NR-
(where R is hydrogen or an organic group); each Y is a
carbonyl, thiocarbonyl, sulphonyl, phosphoryl or similar
acid-forming group: m and n are each zero or 1; and R^1
and R^2 are each hydrogen, an organic group or a group
$-X.Y.(Z)_m-$, or together form a divalent organic group]
may also be useful; as discussed in, for example, WO-A-
9217436 such groups are readily biodegraded in the
presence of esterases, e.g. *in vivo*, but are stable in
the absence of such enzymes. They may therefore
advantageously be linked to therapeutic agents to permit
slow release thereof.

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Poly[N-(2-hydroxyethyl)methacrylamides] are
potentially useful spacer materials by virtue of their
low degree of interaction with cells and tissues (see
e.g. Volfová, I., Ríhová, B. and V.R. and Vetvicka, P.
in *J. Bioact. Comp. Polymers* (1992) 7, 175-190). Work on
a similar polymer consisting mainly of the closely
related 2-hydroxypropyl derivative showed that it was
endocytosed by the mononuclear phagocyte system only to
a rather low extent (see Goddard, P., Williamson, I.,
Bron, J., Hutchkinson, L.E., Nicholls, J. and Petrak, K.
in *J. Bioct. Compat. Polym.* (1991) 6, 4-24.).

Other potentially useful polymeric spacer
materials include:

i)   copolymers of methyl methacrylate with methacrylic
acid; these may be erodible (see Lee, P.I. in *Pharm.
Res.* (1993) 10, 980) and the carboxylate substituents
may cause a higher degree of swelling than with neutral
polymers;

ii)   block copolymers of polymethacrylates with
biodegradable polyesters (see e.g. San Roman, J. and
Guillen-Garcia, P. in *Biomaterials* (1991) 12, 236-241);

iii)   cyanoacrylates, i.e. polymers of esters of 2-
cyanoacrylic acid - these are biodegradable and have
been used in the form of nanoparticles for selective
drug delivery (see Forestier, F., Gerrier, P., Chaumard,
C., Quero, A.M., Couvreur, P. and Labarre, C. in *J.
Antimicrob. Chemoter.* (1992) 30, 173-179);

iv)   polyvinyl alcohols, which are water-soluble and
generally regarded as biocompatible (see e.g. Langer, R.
in *J. Control. Release* (1991) 16, 53-60);

v)   copolymers of vinyl methyl ether with maleic
anhydride, which have been stated to be bioerodible (see
Finne, U., Hannus, M. and Urtti, A. in *Int. J. Pharm.*
(1992) 78. 237-241);

vi)   polyvinylpyrrolidones, e.g. with molecular weight
less than about 25,000, which are rapidly filtered by
the kidneys (see Hespe, W., Meier, A. M. and

Blankwater, Y. M. in *Arzeim.-Forsch./Drug Res. (1977)*
27, 1158-1162);

vii)   polymers and copolymers of short-chain aliphatic
hydroxyacids such as glycolic, lactic, butyric, valeric
and caproic acids (see e.g. Carli, F. in *Chim. Ind.
(Milan)* (1993) **75**, 494-9), including copolymers which
incorporate aromatic hydroxyacids in order to increase
their degradation rate (see Imasaki, K., Yoshida, M.,
Fukuzaki, H., Asano, M., Kumakura, M., Mashimo, T.,
Yamanaka, H. and Nagai. T. in *Int. J. Pharm.* (1992) **81**,
31-38);

viii) polyesters consisting of alternating units of
ethylene glycol and terephthalic acid, e.g. Dacron[R],
which are non-degradable but highly biocompatible;

ix)   block copolymers comprising biodegradable segments
of aliphatic hydroxyacid polymers (see e.g. Younes, H.,
Nataf, P.R., Cohn, D., Appelbaum, Y.J., Pizov, G. and
Uretzky, G. in *Biomater. Artif. Cells Artif. Organs*
(1988) **16**, 705-719), for instance in conjunction with
polyurethanes (see Kobayashi, H., Hyon, S.H. and Ikada,
Y. in "Water-curable and biodegradable prepolymers" - *J.
Biomed. Mater. Res.* (1991) **25**, 1481-1494);

x)   polyurethanes, which are known to be well-
tolerated in implants, and which may be combined with
flexible "soft" segments, e.g. comprising poly(tetra
methylene glycol), poly(propylene glycol) or
poly(ethylene glycol) and aromatic "hard" segments, e.g.
comprising 4,4'-methylenebis(phenylene isocyanate) (see
e.g. Ratner, B.D., Johnston, A.B. and Lenk, T.J. in *J.
Biomed. Mater. Res: Applied Biomaterials* (1987) **21**, 59-
90; Sa Da Costa, V. *et al.* in *J. Coll. Interface Sci.*
(1981) **80**, 445-452 and Affrossman, S. *et al.* in *Clinical
Materials* (1991) **8**, 25-31);

xi)   poly(1,4-dioxan-2-ones), which may be regarded as
biodegradable esters in view of their hydrolysable ester
linkages (see e.g. Song, C. X., Cui, X. M. and
Schindler, A. in *Med. Biol. Eng. Comput.* (1993) **31**,

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S147-150), and which may include glycolide units to
improve their absorbability (see Bezwada, R.S., Shalaby,
S.W. and Newman, H.D.J. in *Agricultural and synthetic
polymers: Biodegradability and utilization* **(1990)** (ed
Glass, J.E. and Swift, G.), 167-174 - ACS symposium
Series, #433, Washington D.C., U.S.A. - American
Chemical Society);

xii)   polyanhydrides such as copolymers of sebacic acid
(octanedioic acid) with bis(4-carboxy-phenoxy)propane,
which have been shown in rabbit studies (see Brem, H.,
Kader, A., Epstein, J.I., Tamargo, R.J., Domb, A.,
Langer, R. and Leong, K.W. in *Sel. Cancer Ther*. (1989)
5, 55-65) and rat studies (see Tamargo, R.J., Epstein,
J.I., Reinhard, C.S., Chasin, M. and Brem, H. in *J.
Biomed. Mater. Res*. (1989) **23**, 253-266) to be useful for
controlled release of drugs in the brain without evident
toxic effects;

xiii) biodegradable polymers containing ortho-ester
groups, which have been employed for controlled release
in vivo (see Maa, Y.F. and Heller, J. in *J. Control.
Release* (1990) **14**, 21-28); and

xiv)   polyphosphazenes, which are inorganic polymers
consisting of alternate phosphorus and nitrogen atoms
(see Crommen, J.H., Vandorpe, J. and Schacht, E.H. in *J.
Control. Release* (1993) **24**, 167-180).

The following tables list linking agents and
agents for protein modification which may be useful in
preparing targetable agents in accordance with the
invention.

Heterobifunctional linking agents

Linking agent	Reactivity 1	Reactivity 2	Comments
ABH	carbohydrate	photoreactive	
ANB-NOS	-NH$_2$	photoreactive	

APDP(1)	-SH	photoreactive	iodinable disulphide linker
APG	-NH$_2$	photoreactive	reacts selectively with Arg at pH 7-8
ASIB(1)	-SH	photoreactive	iodinable
ASBA(1)	-COOH	photoreactive	iodinable
EDC	-NH$_2$	-COOH	zero-length linker
GMBS	-NH$_2$	-SH	
sulfo-GMBS	-NH$_2$	-SH	water-soluble
HSAB	-NH$_2$	photoreactive	
sulfo-HSAB	-NH$_2$	photoreactive	water-soluble
MBS	-NH$_2$	-SH	
sulfo-MBS	-NH$_2$.	-SH	water-soluble
M$_2$C$_2$H	carbohydrate	-SH	
MPBH	carbohydrate	-SH	
NHS-ASA(1)	-NH$_2$	photoreactive	iodinable
sulfo-NHS-ASA(1)	-NH$_2$	photoreactive	water-soluble, iodinable
sulfo-NHS-LC-ASA(1)	-NH$_2$	photoreactive	water-soluble, iodinable
PDPH	carbohydrate	-SH	disulphide linker
PNP-DTP	-NH$_2$	photoreactive	
SADP	-NH$_2$	photoreactive	disulphide linker
sulfo-SADP	-NH$_2$	photoreactive	water-soluble disulphide linker

SAED	-NH$_2$	photoreactive	disulphide linker
SAND	-NH$_2$	photoreactive	water-soluble disulphide linker
SANPAH	-NH$_2$	photoreactive	
sulfo-SANPAH	-NH$_2$	photoreactive	water-soluble
SASD(1)	-NH$_2$	photoreactive	water-soluble iodinable disulphide linker
SIAB	-NH$_2$	-SH	
sulfo-SIAB	-NH$_2$	-SH	water-soluble
SMCC	-NH$_2$	-SH	
sulfo-SMCC	-NH$_2$	-SH	water-soluble
SMPB	-NH$_2$	-SH	
sulfo-SMPB	-NH$_2$	-SH	water-soluble
SMPT	-NH$_2$	-SH	
sulfo-LC-SMPT	-NH$_2$	-SH	water-soluble
SPDP	-NH$_2$	-SH	
sulfo-SPDP	-NH$_2$	-SH	water-soluble
sulfo-LC-SPDP	-NH$_2$	-SH	water-soluble
sulfo-SAMCA(2)	-NH$_2$	photoreactive	
sulfo-SAPB	-NH$_2$	photoreactive	water-soluble

Notes: (1)=iodinable; (2)=fluorescent

Homobifunctional linking agents

Linking agent	Reactivity	Comments
BS	-NH$_2$	
BMH	-SH	
BASED(1)	photoreactive	iodinable disulphide linker

BSCOES	$-NH_2$	
sulfo-BSCOES	$-NH_2$	water-soluble
DFDNB	$-NH_2$	
DMA	$-NH_2$	
DMP	$-NH_2$	
DMS	$-NH_2$	
DPDPB	$-SH$	disulphide linker
DSG	$-NH_2$	
DSP	$-NH_2$	disulphide linker
DSS	$-NH_2$	
DST	$-NH_2$	
sulfo-DST	$-NH_2$	water-soluble
DTBP	$-NH_2$	disulphide linker
DTSSP	$-NH_2$	disulphide linker
EGS	$-NH_2$	
sulfo-EGS	$-NH_2$	water-soluble
SPBP	$-NH_2$	

Biotinylation agents

Agent	Reactivity	Comments
biotin-BMCC	$-SH$	
biotin-DPPE*		preparation of biotinylated liposomes
biotin-LC-DPPE*		preparation of biotinylated liposomes
biotin-HPDP	$-SH$	disulphide linker
biotin-hydrazide	carbohydrate	
biotin-LC-hydrazide	carbohydrate	
iodoacetyl-LC-biotin	$-NH_2$	
NHS-iminobiotin	$-NH_2$	reduced affinity for avidin

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NHS-SS-biotin	-NH₂	disulphide linker
photoactivatable biotin	nucleic acids	
sulfo-NHS-biotin	-NH₂	water-soluble
sulfo-NHS-LC-biotin	-NH₂	

Notes: DPPE=dipalmitoylphosphatidylethanolamine; LC=long chain

Agents for protein modification

Agent	Reactivity	Function
Ellman's reagent	-SH	quantifies/detects/protects
DTT	-S.S-	reduction
2-mercaptoethanol	-S.S-	reduction
2-mercaptylamine	-S.S-	reduction
Traut's reagent	-NH₂	introduces -SH
SATA	-NH₂	introduces protected -SH
AMCA-NHS	-NH₂	fluorescent labelling
AMCA-hydrazide	carbohydrate	fluorescent labelling
AMCA-HPDP	-S.S-	fluorescent labelling
SBF-chloride	-S.S-	fluorescent detection of -SH
N-ethylmaleimide	-S.S-	blocks -SH
NHS-acetate	-NH₂	blocks and acetylates -NH₂
citraconic anhydride	-NH₂	reversibly blocks and introduces negative charges
DTPA	-NH₂	introduces chelator
BNPS-skatole	tryptophan	cleaves tryptophan residue
Bolton-Hunter	-NH₂	introduces iodinable group

Other potentially useful protein modifications include partial or complete deglycosidation by neuraminidase, endoglycosydases or periodate, since deglycosidation often results in less uptake by liver,

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spleen, macrophages etc., whereas neo-glycosylation of
proteins often results in increased uptake by the liver
and macrophages); preparation of truncated forms by
proteolytic cleavage, leading to reduced size and
shorter half life in circulation; and cationisation,
e.g. as described by Kumagi et al. in J. Biol. Chem.
(1987) 262, 15214-15219; Triguero et al. in Proc. Natl.
Acad. Sci. USA (1989) 86, 4761-4765; Pardridge et al. in
J. Pharmacol. Exp. Therap. (1989) 251, 821-826 and
Pardridge and Boado, Febs Lett. (1991) 288, 30-32.
        Vectors which may be usefully employed in
targetable agents according to the invention include the
following:

i)   Antibodies, which can be used as vectors for a
very wide range of targets, and which have advantageous
properties such as very high specificity, high affinity
(if desired), the possiblity of modifying affinity
according to need etc. Whether or not antibodies will
be bioactive will depend on the specific vector/target
combination. Both conventional and genetically
engineered antibodies may be employed, the latter
permitting engineering of antibodies to particular
needs, e.g. as regards affinity and specificity. The
use of human antibodies may be preferred to avoid
possible immune reactions against the vector molecule.
A further useful class of antibodies comprises so-called
bi- and multi-specific antibodies, i.e. antibodies
having specificity for two or more different antigens in
one antibody molecule. Such antibodies may, for
example, be useful in promoting formation of bubble
clusters and may also be used for various therapeutic
purposes, e.g. for carrying toxic moieties to the
target. Various aspects of bispecific antibodies are
described by McGuinness, B.T. et al. in Nat. Biotechnol.
(1996) 14, 1149-1154; by George, A.J. et al. in J.
Immunol. (1994) 152, 1802-1811; by Bonardi et al. in

Cancer Res. (1993) **53**, 3015-3021; and by French, R.R. *et al.* in *Cancer Res.* (1991) **51**, 2353-2361.

ii)   Cell adhesion molecules, their receptors,
cytokines, growth factors, peptide hormones and pieces
thereof. Such vectors rely on normal biological
protein-protein interactions with target molecule
receptors, and so in many cases will generate a
biological response on binding with the targets and thus
be bioactive; this may be a relatively insignificant
concern with vectors which target proteoglycans.

iii)   Non-peptide agonists/antagonists or non-bioactive
binders of receptors for cell adhesion molecules,
cytokines, growth factors and peptide hormones. This
category may include non-bioactive vectors which will be
neither agonists nor antagonist but which may
nonetheless exhibit valuable targeting ability.

iv)   Oligonucleotides and modified oligonucleotides
which bind DNA or RNA through Watson-Crick or other
types of base-pairing. DNA is usually only present in
extracelluar space as a consequence of cell damage, so
that such oligonucleotides, which will usually be non-
bioactive, may be useful in, for example, targeting of
necrotic regions, which are associated with many
different pathological conditions. Oligonucleotides may
also be designed to bind to specific DNA- or RNA-binding
proteins, for example transcription factors which are
very often highly overexpressed or activated in tumour
cells or in activated immune or endothelial cells.
Combinatorial libraries may be used to select
oligonucleotides which bind specifically to any possible
target molecules and which therefore may be employed as
vectors for targeting.

v)   DNA-binding drugs may behave similarly to

oligonuclotides, but may exhibit biological acitvity and/or toxic effects if taken up by cells.

vi)   Protease substrates/inhibitors. Proteases are involved in many pathological conditions. Many substrates/inhibitors are non-peptidic but, at least in the case of inhibitors, are often bioactive.

vii)   Vector molecules may be generated from combinatorial libraries without necessarily knowing the exact molecular target, by functionally selecting (in vitro, ex vivo or in vivo) for molecules binding to the region/structure to be imaged.

viii) Various small molecules, including bioactive compounds known to bind to biological receptors of various kinds. Such vectors or their targets may be used for generate non-bioactive compounds binding to the same targets.

ix).   Proteins or peptides which bind to glucosamioglycan side chains e.g. heparan sulphate, including glucosoaminoglycan-binding portions of larger molecules, as binding to glucosoaminoglycans does not result in a biological response. Proteoglycans are not found on red blood cells, which eliminates undesirable adsorption to these cells.

        Other peptide vectors and lipopeptides thereof of particular interest for targeted ultrasound imaging are listed below: Atherosclerotic plaque binding peptides such as YRALVDTLK, YAKFRETLEDTRDRMY and RALVDTEFKVKQEAGAK; Thrombus binding peptides such as NDGDFEEIPEEYLQ and GPRG, Platelet binding peptides such as PLYKKIIKKLLES; and cholecystokinin, α-melanocyte-stimulating hormone, heat stable enterotoxin 1, vasoactive intestinal peptide, synthetic alpha-M2

peptide from the third heavy chain complementarity-
determininig region and analogues thereof for tumour
targeting.

The following tables identify various vectors
which may be targeted to particular types of targets and
indicated areas of use for targetable diagnostic and/or
therapeutic agents according to the invention which
contain such vectors.

Protein and peptide vectors - antibodies

Vector type	Target	Comments/areas of use	Ref
antibodies (general)	CD34	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells	1
"	ICAM-1	"	1
"	ICAM-2	"	1
"	ICAM-3	"	1
"	E-selectin	"	1
"	P-selectin	"	1
"	PECAM	"	1
"	Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	"	2
"	GlyCAM	Vessel wall in lymph nodes (quite specific for lymph nodes)	3
"	MadCam 1	"	3

	fibrin	Thrombi	4
"	Tissue Factor	Activated endothelium, tumours	5
"	Myosin	Necrosis, myocardial infaction	6
"	CEA (carcino-embryonal antigen)	Tumours	7
"	Mucins	Tumours	8
"	Multiple drug resistance protein	Tumours	9
"	Prostate specific antigen	Prostate cancer	
"	Cathepsin B	Tumours (proteases of various kinds are often more or less specifically overexpressed in a variety of tumours - Cathepsin B is such a protease)	10
"	Transferrin receptor	Tumors, vessel wall	11
MoAb 9.2.27		Tumours Antigen upregulated on cell growth	12
	VAP-1	Adhesion molecule	13

		Band 3 protein	Upregulated during phagocytic activity	
	antibodies	CD34(sialomucin)	endothelial cells	
	antibodies	CD31(PECAM-1)	endothelial cells	
	antibodies	intermediate filaments necrotic cells/tissue		
	antibodies	CD44	tumour cells	a
	antibodies	ß2-micro-globulin	general	b
	antibodies	MHC class 1	general	b
	antibodies	integrin $\alpha v \beta 3$	tumours; angiogenesis	c

References

a)   Heider, K. H., M. Sproll, S. Susani, E. Patzelt, P. Beaumier, E. Ostermann, H. Ahorn, and G. R. Adolf. **1996.** "Characterization of a high-affinity monoclonal antibody specific for CD44v6 as candidate for immunotherapy of squamous cell carcinomas". *Cancer Immunology Immunotherapy* **43**: 245-253.

b)   I. Roitt, J. Brostoff, and D. Male. **1985.** *Immunology*, London: Gower Medical Publishing, p. 4.7

c)   Stromblad, S., and D. A. Cheresh. **1996.** "Integrins, angiogenesis and vascular cell survival". *Chemistry & Biology* **3**: 881-885.

Protein and peptide vectors - cell adhesion molecules etc.

Vector type	Target	Comments/areas of use	Ref
L-selectin	CD34 MadCAM1 GlyCam 1	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, Lymph nodes	3
Other selectins	carbohydrate ligands (sialyl Lewis x) heparan sulfate	vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium	14
RGD-peptides	integrins	"	2
PECAM	PECAM, and other	Endothelium, Cells in immune system	15
Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	Laminin, collagen, fibronectin, VCAM-1, thrombospondin, vitronectin etc.	Endothelium, Vessel wall etc.	16
Integrin receptors, e.g.Laminin, collagen, fibronectin, VCAM-1, thrombospondin, vitronectin etc.	Integrins, e.g. VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, $\beta_1\alpha_7$, $\beta_1\alpha_8$, $\beta_1\alpha_v$, LFA-1, Mac-1, CD41a, etc.	Cells in immune system vessel wall etc.	17 18

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Nerve cell adhesion molecule (N-CAM)	proteoglycans N-CAM (homophilic)		19
integrin αvβ3	CD31 (PECAM-1)	endothelial cells	
RGD-peptides	integrins	angiogenesis	c

Vectors comprising cytokines/growth factors/peptide hormones and fragments thereof

Vector type	Target	Comments/areas of use	Ref
Epidermal growth factor	EGF-receptor or related receptors	Tumours	20
Nerve growth factor	NGF-receptor	Tumours	21
Somatostatin	ST-receptor	Tumours	22
Endothelin	Endothelin-receptor	Vessel wall	
Interleukin-1	IL-1-receptor	Inflammation, activated cells of different kinds	23
Interleukin-2	IL-2-receptor	"	24
Chemokines (ca. 20 different cytokines partly sharing receptors)	Chemokine receptors, proteoglycans	Inflammation	25
Tumour necrosis factor	TNF-receptors	Inflammation	
Parathyroid hormone	PTH-receptors	Bone diseases Kidney diseases	

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Bone Morphogenetic Protein	BMP-receptors	Bone Diseases	
Calcitonin	CT-receptors	Bone diseases	
Colony stimulating factors (G-CSF, GM-CSF, M-CSF, IL-3)	Corresponding specific receptors, proteoglycans	Endothelium	26
Insulin like growth factor I	IGF-I receptor	Tumours, other growing tissues	
Atrial Natriuretic Factor	ANF-receptors	Kidney, vessel wall	
Vasopressin	Vasopressin receptor	Kidney, vessel wall	
VEGF	VEGF-receptor	Endothelium, regions of angiogenesis	
Fibroblast growth factors	FGF-receptors, Proteoglycans	Endothelium Angiogenesis	27
Schwann cell growth factor	proteoglycans specific receptors		28

Miscellaneous protein and peptide vectors

Vector type	Target	Comments/areas of use	Ref
Streptavidin	Kidney	Kidney diseases	29
Bacterial fibronectin-binding proteins	Fibronectin	Vessel wall	30

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Fc-part of antibodies	Fc-receptors	Monocytes macrophages liver	31
Transferrin	transferrin-receptor	Tumours vessel walls	11
Streptokinase/ tissue plasminogen activator	thrombi	thrombi	
Plasminogen, plasmin	Fibrin	Thrombi, tumours	32
Mast cell proteinases	proteoglycans		33
Elastase	proteoglycans		34
Lipoprotein lipase	proteoglycans		35
Coagulation enzymes	proteoglycans		36
Extracellular superoxide dismutase	proteoglycans		37
Heparin cofactor II	proteoglycans		38
Retinal survival factor	proteoglycans specific receptors		39
Heparin-binding brain mitogen	proteoglycans specific receptors		40
Apolipoprotein, e.g. apolipoprotein B	proteoglycans specific receptors (e.g., LDL receptor)		41

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Apolipoprotein E	LDL receptor proteoglycans		42
Adhesion-promoting proteins, e.g. Purpurin	proteoglycans		43
Viral coat proteins, e.g. HIV, Herpes	proteoglycans		44
Microbial adhesins, e.g. "Antigen 85" complex of mycobacteria	fibronectin, collagen, fibrinogen, vitronectin, heparan sulfate		45
ß-amyloid precursor	proteoglycans	ß-amyloid accumulates in Alzheimer's disease	46
Tenascin, e.g .tenascin C	heparan sulfate, integrins		47

Vectors comprising non-peptide agonists/antagonists or non-bioactive binders of receptors for cytokines/growth factors/peptide hormones/cell adhesion molecules

Vector type	Target	Comments/areas of use	Ref
		Several agonists/antagonists are known for such factors acting through G-protein coupled receptors	48 49
Endothelin antagonist	Endothelin receptor	Vessel wall	
Desmopressin (vasopressin analogue)	Vasopressin receptor	Kidney Vessel wall	

Demoxytocin (oxytocin analogue)	Oxytocin Receptor	Reproductive organs, Mammary glands, Brain	
Angiotensin II receptor antagonists CV-11974, TCV-116	Angiotensin II receptors	Vessel wall brain adrenal gland	
non-peptide RGD-analogues	integrins	Cells in immune system vessel wall etc.	50

Vectors comprising anti-angiogenic factors

Vector type	Target	Comments/areas of use	Ref
Angiostatin	EC of tumors	plasminogen fragment	K
cartilage-derived inhibitor	EC of tumors		J
β-Cyclodextrin tetradecasulfate	tumors, inflammation		C
fumagillin and analogs	tumors, inflammation		E
Interferon-α	EC of tumors		K
Interferon-γ	EC of tumors		E
interleukin-12	EC of tumors		E
linomide	tumors, inflammation		A
medroxyprogesterone	EC of tumors		K
metalloproteinase inhibitors	EC of tumors		K
pentosan polysulfate	EC of tumors		K
platelet factor 4	EC of tumors		M
Somatostatin	EC of tumors		K

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Suramin	EC of tumors		K
Taxol	EC of tumors		K
thalidomide	EC of tumors		K
Thrombospondin	EC of tumors		K

5

Vectors comprising angiogenic factors

Vector type	Target	Comments/areas of use	Ref
acidic fibroblast growth factor	EC of tumors		K
adenosine	EC of tumors		K
Angiogenin	EC of tumors		K
Angiotensin II	EC of tumors		K
basement membrane components	tumors	e.g., tenascin, collagen IV	M
basic fibroblast growth factor	EC of tumors		K
Bradykinin	EC of tumors		K
Calcitonin gene-related peptide	EC of tumors		K
epidermal growth factor	EC of tumors		K
Fibrin	tumors		K
Fibrinogen	tumors		K
Heparin	EC of tumors		K
histamine	EC of tumors		K
hyaluronic acid or fragments thereof	EC of tumors		K
Interleukin-1α	EC of tumors		K
laminin, laminin fragments	EC of tumors		K
nicotinamide	EC of tumors		K
platelet activating factor	EC of tumors		K

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Platelet-derived endothelial growth factor	EC of tumors		K
prostaglandins E1, E2	EC of tumors		K
spermine	EC of tumors		K
spermine	EC of tumors		K
Substance P	EC of tumors		K
transforming growth factor-α	EC of tumors		K
transforming growth factor-β	EC of tumors		K
Tumor necrosis factor-α	EC of tumors		K
vascular endothelial growth factor/vascular permeability factor	EC of tumors		K
vitronectin			A

Vector molecules other than recognized angiogenetic factors with known affinity for receptors associated with angiogenesis

Vector type	Target	Comments/areas of use	Ref
angiopoietin	tumors, inflammation		B
α₂-antiplasmin	tumors, inflammation		
combinatorial libraries, compounds from	tumors, inflammation	for instance: compounds that bind to basement membrane after degradation	
endoglin	tumors, inflammation		D
endosialin	tumors, inflammation		D
endostatin [collagen fragment]	tumors, inflammation		M

Factor VII related antigen	tumors, inflammation		D
fibrinopeptides	tumors, inflammation		ZC
fibroblast growth factor, basic	tumors, inflammation		E
hepatocyte growth factor	tumors, inflammation		I
insulin-like growth factor	tumors, inflammation		R
interleukins	tumors, inflammation	e.g.,: IL-8	I
leukemia inhibitory factor	tumors, inflammation		A
metalloproteinase inhibitors	tumors, inflammation	e.g., batimastat	E
Monoclonal antibodies	tumors, inflammation	for instance: to angiogenetic factors or their receptors, or to components of the fibrinolytic system	
peptides, for instance cyclic RGDᴅFV	tumors, inflammation		B,Q
placental growth factor	tumors, inflammation		J
placental proliferin-related protein	tumors, inflammation		E
plasminogen	tumors, inflammation		M
plasminogen activators	tumors, inflammation		D

plasminogen activator inhibitors	tumors, inflammation		U,V
platelet activating factor antagonists	tumors, inflammation	inhibitors of angiogenesis	A
platelet-derived growth factor	tumors, inflammation		E
pleiotropin	tumors, inflammation		ZA
proliferin	tumors, inflammation		E
proliferin related protein	tumors, inflammation		E
selectins	tumors, inflammation	e.g., E-selectin	D
SPARC	tumors, inflammation		M
snake venoms (RGD-containing)	tumors, inflammation		Q
Tissue inhibitor of metalloproteinases	tumors, inflammation	e g,, TIMP-2	U
thrombin	tumors, inflammation		H
thrombin-receptor-activating tetradecapeptide	tumors, inflammation		H
thymidine phosphorylase	tumors, inflammation		D
tumor growth factor	tumors, inflammation		ZA

Receptors/targets associated with angiogenesis

Vector type	Target	Comments/areas of use	Ref
biglycan	tumors, inflammation	dermatan sulfate proteoglycan	X
CD34	tumors, inflammation		L
CD44	tumors, inflammation		F
collagen type I, IV, VI, VIII	tumors, inflammation		A
decorin	tumors, inflammation	dermatan sulfate proteoglycan	Y
dermatan sulfate proteoglycans	tumors, inflammation		X
endothelin	tumors, inflammation		G
endothelin receptors	tumors, inflammation		G
fibronectin	tumors		P
Flk-1/KDR, Flt-4	tumors, inflammation	VEGF receptor	D
FLT-1 (fms-like tyrosine kinase)	tumors, inflammation	VEGF-A receptor	O
heparan sulfate	tumors, inflammation		P
hepatocyte growth factor receptor (c-met)	tumors, inflammation		I
insulin-like growth factor/mannose-6-phosphate receptor	tumors, inflammation		R

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integrins: β₃ and β₅, integrin αᵥβ₃, integrin α₆β₁, , integrins α₆, integrins β₁, integrin α₂β₁, integrin αᵥβ₃, integrin α₅ integrin αᵥβ₅, fibrin receptors.	Tumors, inflammation	laminin receptor subunit of the fibronectin receptor	D, P
Intercellular adhesion molecule-1 and -2	tumors, inflammation		P
Jagged gene product	tumors, inflammation		T
Ly-6	tumors, inflammation	a lymphocyte activation protein	N
matrix metalloproteinases	tumors, inflammation		D
MHC class II	tumors, inflammation		
Notch gene product	tumors, inflammation		T
Osteopontin	tumors		Z
PECAM	tumors, inflammation	alias CD31	P
plasminogen activator receptor	tumors, inflammation		ZC
platelet-derived growth factor receptors	tumors, inflammation		E
Selectins: E-, P-	tumors, inflammation		D

Sialyl Lewis-X	tumors, inflammation	blood group antigen	M
stress proteins: glucose regulated, heat shock families and others	tumors, inflammation	molecular chaperones	
syndecan	tumors, inflammation		T
thrombospondin	tumors, inflammation		M
TIE receptors	tumors, inflammation	tyrosine kinases with Ig- and EGF-Iike domains	E
tissue factor	tumors, inflammation		Z
tissue inhibitor of metalloproteinases	tumors, inflammation	e.g., TIMP-2	U
transforming growth factor receptor	tumors, inflammation		E
urokinase-type plasminogen activator receptor	tumors, inflammation		D
Vascular cellular adhesion molecule (VCAM)	tumors, inflammation		D
Vascular endothelial growth factor related protein	tumors, inflammation		
Vascular endothelial growth factor-A receptor	tumors, inflammation		K
von Willebrand factor-related antigen	tumors, inflammation		L

Oligonucleotide vectors

	Vector type	Target	Comments/areas of use	Ref
5 10	Oligonucleotides complementary to repeated sequences, e.g. genes for ribosomal RNA, Alu-sequences	DNA made available by necrosis	Tumours Myocardial infarction All other diseases that involves necrosis	51
15	Oligonucleotides complementary to disease-specific mutations (e.g. mutated oncogenes).	DNA made available by necrosis in a region of the relevant disease	Tumours	51
20	Oligonucleotides complementary to DNA of infecting agent.	DNA of infective agent	Viral or bacterial infections	51
	Triple or quadruple-helix forming oligonucleotides	As in above examples	As in above examples	51
25 30	Oligonucleotides with recognition sequence for DNA-or RNA-binding proteins	DNA-binding protein, e.g. transcription factors (often overexpressed/ activated in tumours or activated endothelium/ immune cells	Tumours Activated endothelium Activated immune cells	

Modified oligonucleotide vectors

	Vector type	Target	Comments/areas of use	Ref
5	Phosphorothioate oligos	As for unmodified oligos	As for unmodified oligos	51
	2'-O-methyl substituted oligos	"	"	51
	circular oligos	"	"	51
10	oligos containing hairpin structure to decrease degradation	"	"	51
15	oligos with terminal phosphorothioate	"	"	51
	2'-fluoro oligos	"	"	51
20	2'-amino oligos	"	"	51
	DNA-binding drugs conjugated to oligos (for examples, see below)	"	Increased binding affinity as compared to pure oligos	52
25				
30	Peptide Nucleic Acids (PNAs, oligonucleotidss with a peptide backbone)	"	Increased binding affinity and stability compared to standard oligos.	53

Nucleoside and nucleotide vectors

Vector type	Target	Comments/areas of use	Ref
Adenosine or analogues	Adenosine receptors	Vessel wall Heart	54
ADP, UDP, UTP and others	Various nucleotide receptors	Many tissues, e.g. brain, spinal cord, kidney, spleen	55

Receptors comprising DNA-binding drugs

Vector type	Target	Comments/areas of use	Ref
acridine derivatives distamycin netropsin actinomycin D echinomycin bleomycin etc.	DNA made available by necrosis	Tumours, Myocardial infarction and all other diseases involving necrosis or other processes liberating DNA from cells	

Receptors comprising protease substrates

Vector type	Target	Comments/areas of use	Ref
Peptidic or non-peptidic substrates	Cathepsin B	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds, e.g. Cathepsin B	10

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Receptors comprising protease inhibitors

Vector type	Target	Comments/areas of use	Ref
Peptidic or non-peptidic inhibitors e.g. N-acetyl-Leu-Leu-norleucinal	Cathepsin B	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds, e.g. Cathepsin B	10
bestatin ([(2S,3R)-3-Amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine hydrochloride)	Aminopeptidases	Tumours, e.g. on cell surfaces	
Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride)	Serine proteases	Tumours, vessel wall etc.	
Commercially available inhibitors e.g. kaptopril enalapril ricionopril	Angiotensin converting enzyme	Endothelial cells	
Low specificity non-peptidic compounds	Coagulation factors	Vessel wall injury, tumours, etc.	
Protease nexins (extracellular protease inhibitors)	proteoglycans		56

Antithrombin	proteoglycans, Coagulation factors		57

Vectors from combinatorial libraries

Vector type	Target	Comments/areas of use	Ref
Antibodies with structure determined during generation process	Any of above targets - or may be unknown when make functional selection of vector binding to chosen diseased structure	Any diseased or normal structure of interest, e.g. thrombi, tumours or walls of myocardial vessels	58, 59, 60
Peptides with sequence determined during generation process	"	"	58, 59, 60
Oligonucleotides with sequence determined during generation process	"	"	58, 59, 60
Modifications of oligos obtained as above	"	"	58, 59, 60

Other chemicals with structure determined during generation process	"	"	58, 59, 60

Carbohydrate vectors

Vector type	Target	Comments/areas of use	Ref
neo-glycoproteins	macrophages	general activation/ inflammation	
oligosaccharides with terminal galactose	Asialo-glycoprotein receptor	liver	61
Hyaluronan	aggrecan (a proteoglycan) "link proteins" cell-surface receptors: CD44		62
Mannose		Blood brain barrier, Brain tumours and other diseases causing changes in BBB	63
Bacterial glycopeptides		"	64

(Glyco)Lipid vectors

Vector type	Target	Comments/areas of use	Ref
GM1 gangliosides	cholera bacteria in the gastrointestinal tract	diagnosis/treatment of cholera	

platelet activating factor (PAF) antagonists	PAF receptors	diagnosis of inflammation	
Prostoglandin antagonists of inflammation	Prostoglandin receptors	diagnosis of inflammation	
Thromboxane antagonists of inflammation	Leukotriene receptors	diagnosis of inflammation	

Small molecule vectors

Vector type	Target	Comments/areas of use	Ref
Adrenalin	Corresponding receptors		
Betablockers	Adrenergic beta-receptors	Myocardium for beta-1 blockers	
Alpha-blockers	Adrenergic alpha-receptors	Vessel wall	
benzodiazepines			
serotonin-analogues	Serotonin-receptors		
anti-histamines	Histamine-receptors	Vessel wall	
Acetyl-choline receptor antagonists	ACh-receptors		
verapamil	Ca^{2+}-channel blocker	Heart muscle	
nifedipin	Ca^{2+}-channel blocker	Heart muscle	

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Amiloride	Na$^+$/H$^+$-exchanger	Blocks this exchanges in kidney and is generally upregulated in cells stimulated by growth factors.	
Digitalis glycosides	Na$^+$/K$^+$-ATP-ases	myocardium peripheral vasculature, central nervous system	
Thromboxae/ Prostaglandin receptor antagonists or agonists	Thromboxane/ prostaglandin receptors	Vessel wall, Endothelium	
Glutathione	Glutathione-receptors Leukotriene-receptors	Lung, Brain	
Biotin	biotin transport protein on cell surface		65
Folate	folate transport protein on cell surface	Tumours	66
Riboflavin	riboflavin transport protein on cell surface		67

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The following non-limitative examples serve to illustrate the invention. Confirmation of the microparticulate nature of products is performed using microscopy as described in WO-A-9607434. Ultrasonic transmission measurements may be made using a broadband transducer to indicate microbubble suspensions giving an increased sound beam attenuation compared to a standard. Flow cytometric analysis of products can be used to confirm attachment of macromolecules thereto. The ability of targeted microbubbles to bind specifically to cells expressing a target may be studied *in vitro* by microscopy and/or using a flow chamber containing immobilised cells, for example employing a population of cells expressing the target structure and a further population of cells not expressing the target. Radioactive, fluorescent or enzyme-labelled streptavidin/avidin may be used to analyse biotin attachment.

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Example 1 - Adhesion of poly-L-lysine-coated
phosphatidylserine-encapsulated microbubbles to
endothelial cells

5   Poly-L-lysine (8 mg) having a molecular weight of 115
     kDa was dissolved in water (400 μl). Freshly
     redispersed microbubbles of phosphatidylserine-
     encapsulated perfluorobutane (40 μl) were incubated in
     either water (400 μl) or the poly-L-lysine solution for
10   15 minutes at room temperature. Zeta potential
     measurements confirmed that the poly-L-lysine-coated
     microbubbles were positively charged while the uncoated
     bubbles were negatively charged. A cell adhesion study
     using human endothelial cells grown in culture dishes
15   was performed with the above-described microbubbles, the
     uncoated microbubbles being used as a control.
     Microscopy of the endothelial cells after incubation
     showed a much increased number of poly-L-lysine-coated
     microbubbles adhering to endothelial cells in comparison
20   to the uncoated microbubbles.

     Example 2 - Gas-filled microbubbles comprising
     phosphatidylserine and RGDC-Mal-PEG$_{3400}$-DSPE
25
     a) Synthesis of Boc-NH-PEG$_{3400}$-DSPE (t-butyl carbamate
     poly(ethylene glycol)distearoylphosphatidyl-
     ethanolamine)

30   DSPE (distearoylphosphatidylethanolamine) (31 mg, Sygena
     Inc.) was added to a solution of Boc-NH-PEG$_{3400}$-SC (t-
     butyl carbamate poly(ethylene glycol)-succinimidyl
     carbonate) (150 mg) in chloroform (2 ml), followed by
     triethylamine (33 μl). The mixture formed a clear
35   solution after stirring at 41 °C for 10 minutes. The
     solvent was rotary evaporated and the residue taken up
     in acetonitrile (5 ml). The thus-obtained dispersion

was cooled to 4 °C and centrifuged, whereafter the
solution was separated from the undissolved material and
evaporated to dryness. The structure of the resulting
product was confirmed by NMR.

b)   Synthesis of H₂N-PEG₃₄₀₀-DSPE (amino-poly(ethylene
glycol)-distearoylphosphatidylethanolamine)

Boc-NH-PEG₃₄₀₀-DSPE (167 mg) was stirred in 4 M
hydrochloric acid in dioxane (5 ml) for 2.5 hours at ·
ambient temperature. The solvent was removed by rotary
evaporation and the residue was taken up in chloroform
(1.5 ml) and washed with water (2 x 1.5 ml). The
organic phase was removed by rotary evaporation. TLC
(chloroform/methanol/water 13:5:0.8) gave the title
product with Rf = 0.6; the structure of the product,
which was ninhydrin positive, was confirmed by NMR.

c)   Synthesis of Mal-PEG₃₄₀₀-DSPE (3-maleimidopropionate
poly(ethylene glycol)distearoylphosphatidyl-
ethanolamine)

A solution of N-succinimidyl-3-maleimidopropionate (5.6
mg, 0.018 mmol) in tetrahydrofuran (0.2 ml) is added to
H₂N-PEG₃₄₀₀-DSPE (65 mg, 0.012 mmol) dissolved in
tetrahydrofuran (1 ml) and 0.1 M sodium phosphate buffer
pH 7.5 (2 ml). The reaction mixture is heated to 30 °C
and the reaction is followed to completion by TLC,
whereafter the solvent is evaporated.

d)   Synthesis of RGDC-Mal-PEG₃₄₀₀-DSPE

Mal-PEG₃₄₀₀-DSPE (0.010 mmol) in 0.1 M sodium phosphate
buffer having a pH of 7.5 is added to the peptide RGDC
(0.010 mmol). The reaction mixture is heated to 37 °C if
necessary and the reaction is followed by TLC to
completion, whereafter the solvent is removed.

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e) Preparation of gas-filled microbubbles encapsulated by phosphatidylserine and RGDC-Mal-PEG$_{3400}$-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%) and Mal-PEG$_{3400}$-DSPE (10-0.1mol%) is added 5% propylene glycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is then transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with 0.1 M sodium phosphate buffer having a pH of 7.5. The peptide RGDC, dissolved in 0.1 M sodium phosphate buffer having a pH of 7.5, is added to the washed microbubbles, which are placed on the roller table. The washing procedure is then repeated.

f) Alternative preparation of gas-filled microbubbles encapsulated by phosphatidylserine and RGDC-Mal-PEG$_{3400}$-DSPE

To phosphatidylserine (5 mg) is added 5% propylene glycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with 0.1 M sodium phosphate buffer having a pH of 7.5. RGDC-Mal-PEG$_{3400}$-DSPE dissolved in 0.1 M sodium phosphate buffer having a pH of 7.5 is added to the washed microbubbles, which are then placed on the roller table. The washing procedure is repeated following incorporation of the RGDC-Mal-PEG$_{3400}$-DSPE into the microbubble membranes.

Example 3 - Gas-filled microbubbles encapsulated with
phosphatidylserine, phosphatidylcholine and biotin-
amidocaproate-PEG$_{3400}$-Ala-cholesterol

a) Synthesis of Z-Ala-cholesterol (3-O-(carbobenzyloxy-
L-alanyl)cholesterol)

Cholesterol (4 mmol), Z-alanine (5 mmol) and
dimethylaminopyridine (4 mmol) were dissolved in
dimethylformamide/tetrahydrofuran (20 ml + 5 ml) and
dicyclohexylcarbodiimide was added. The reaction
mixture was stirred at ambient temperature overnight.
Dicyclohexylurea was filtered off and the solvent was
rotary evaporated. The residue was taken up in
chloroform, undissolved dicyclohexylurea was filtered
off and the solvent was removed by rotary evaporation.
The residue was placed on a column of silica gel, and Z-
Ala-cholesterol was eluted with toluene/petroleum ether
(20:2) followed by toluene/diethyl ether (20:2). The
fractions containing the title compound were combined
and the solvent was removed by rotary evaporation. The
structure of the product was confirmed by NMR.

b) Synthesis of Ala-cholesterol (3-O-(L-alanyl)-
cholesterol)

Z-Ala-cholesterol (0.48 mmol) is placed in
tetrahydrofuran (20 ml) and glacial acetic acid (3 ml)
and hydrogenated in the presence of 5 % palladium on
charcoal for 2 hours. The reaction mixture is filtered
and concentrated *in vacuo*.

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c) Synthesis of Boc-NH-PEG$_{3400}$-Ala-cholesterol

Ala-cholesterol is added to a solution of Boc-NH-PEG$_{3400}$-SC (t-butyl carbamate poly(ethylene glycol)-

5   succinimidyl carbonate) in chloroform, followed by triethylamine. The suspension is stirred at 41 °C for 10 minutes. The crude product is purified by chromatography.

10   ### d) Synthesis of H$_2$N-PEG$_{3400}$-Ala-cholesterol

Boc-NH-PEG$_{3400}$-Ala-cholesterol is stirred in 4 M hydrochloric acid in dioxane for 2.5 hours at ambient temperature. The solvent is removed by rotary

15   evaporation and the residue is taken up in chloroform and washed with water. The organic phase is rotary evaporated to dryness. The crude product may be purified by chromatography.

20   ### e) Synthesis of biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol

A solution of biotinamidocaproate N-hydroxysuccinimide ester in tetrahydrofuran is added to H$_2$N-PEG$_{3400}$-Ala-

25   cholesterol dissolved in tetrahydrofuran and 0.1 M sodium phosphate buffer having a pH of 7.5 (2 ml). The reaction mixture is heated to 30 °C and the reaction is followed to completion by TLC, whereafter the solvent is evaporated.

30

f) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol

35   To a mixture (5 mg) of phosphatidylserine and phosphatidylcholine (in total 90-99.9 mol%) and biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol (10-0.1 mol%)

is added 5% propylene glycol-glycerol in water (1 ml).
The dispersion is heated to not more than 80 °C for 5
minutes and then cooled to ambient temperature. The
dispersion (0.8 ml) is then transferred to a vial (1 ml)

5   and the head space is flushed with perfluorobutane. The
vial is shaken in a cap-mixer for 45 seconds, whereafter
the sample is put on a roller table. After
centrifugation the infranatant is exchanged with water
and the washing is repeated.

10

g) Alternative preparation of gas-filled microbubbles
encapsulated with phosphatidylserine,
phosphatidylcholine and biotinamidocaproate-PEG_{3400}-Ala-
cholesterol

15

To a mixture (5 mg) of phosphatidylserine and
phosphatidylcholine is added 5% propylene glycol-
glycerol in water (1 ml). The dispersion is heated to
not more than 80 °C for 5 minutes and then cooled to

20   ambient temperature. The dispersion (0.8 ml) is then
transferred to a vial (1 ml) and the head space is
flushed with perfluorobutane. The vial is shaken in a
cap-mixer for 45 seconds, whereafter the sample is put
on a roller table. After centrifugation the infranatant

25   is exchanged with water. Biotinamidocaproate-PEG_{3400}-Ala-
cholesterol dissolved in water is added to the washed
microbubbles, which are placed on a roller table for
several hours. The washing procedure is repeated
following incorporation of the biotinamidocaproate-

30   PEG_{3400}-Ala-cholesterol into the microbubble membranes.

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Example 4 - Gas-filled microbubbles comprising
phosphatidylserine, phosphatidylcholine, biotin-
amidocaproate-PEG$_{3400}$-Ala-Cholesterol and drug-cholesterol

a)   Synthesis of drug-cholesterol

Cholesterol (4 mmol), a drug having an acid group and
dimethylaminopyridine (4 mmol) are dissolved in
dimethylformamide/tetrahydrofuran (20 ml + 5 ml) and
dicyclohexylcarbodiimide is added. The reaction mixture
is stirred at ambient temperature overnight.
Dicyclohexylurea is filtered off and the solvent is
rotary evaporated. The title compound is purified by
chromatography.

b)   Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine, phosphatidylcholine,
biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol and drug-
cholesterol

To a mixture (5 mg) of phosphatidylserine and
phosphatidylcholine (in total 90-99.9mol%) and
biotinamidocaproate-PEG$_{3400}$-Ala-cholesterol (prepared as
in Example 3) and drug-cholesterol (in total 10-0.1mol%)
is added 5% propylene glycol-glycerol in water (1 ml).
The dispersion is heated to not more than 80 °C for 5
minutes and then cooled to ambient temperature. The
dispersion (0.8 ml) is transferred to a vial (1 ml) and
the head space is flushed with perfluorobutane. The
vial is shaken in a cap-mixer for 45 seconds whereafter
the sample is put on a roller table. After
centrifugation the infranatant is exchanged with water
and the washing is repeated.

Example 5 - Gas-filled microbubbles encapsulated with phosphatidylserine and thiolated-anti-CD34-Mal-PEG$_{3400}$-DSPE

5   a)   Preparation of thiolated anti-CD34 antibodies

Thiolation of anti-CD34 antibodies may be effected as described by Hansen, C.B. et al.(1995) *Biochim. Biophys. Acta* **1239**, 133-144.

10

b)   Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and thiolated-anti-CD34-Mal-PEG$_{3400}$-DSPE

15   To a mixture (5 mg) of phosphatidylserine (90-99.9mol%) and Mal-PEG$_{3400}$-DSPE (10-0.1mol%, prepared as in Example 2) is added 5% propylene glycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The
20   dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with an
25   appropriate buffer and coupling of the thiolated antibody to the microbubbles is performed, e.g. as described by Goundalkar, A., Ghose, T. and Mezei, M. in *J. Pharm. Pharmacol.* (1984) **36** 465-66 or Hansen, C.B. et al.(1995) *Biochim. Biophys. Acta* **1239** 133-144. The
30   microbubbles are then placed on a roller table for several hours and are washed. Flow cytometric analysis of the resulting microbubbles (employing a fluorescently labeled secondary antibody) is used to confirm attachment of the anti-CD34 antibody to the bubbles.
35   The ability of the bubbles to bind specifically to CD34-expressing cells is studied by microscopy employing one population of cells expressing CD34 and one population

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that do not express CD34.

Example 6 - Biotin attached to gas-filled microbubbles

Biotin may be attached to microbubbles in many different
ways, e.g. in a similar way to that described by Corley,
P. and Loughrey, H.C. in (1994) *Biochim. Biophys. Acta*
1195, 149-156. The resulting bubbles are analysed by
flow cytometry, e.g. by employing fluorescent
streptavidin to detect attachment of biotin to the
bubbles. Alternatively radioactive or enzyme-labelled
streptavidin/avidin is used to analyse biotin
attachment.

Example 7 - Gas-filled microbubbles encapsulated with
distearoylphosphatidylserine and biotin-DPPE

To distearoylphosphatidylserine (DSPS) (22.6 mg) was
added 4% propylene glycol-glycerol in water (4 ml). The
dispersion was heated to not more than 80 °C for five
minutes and then cooled to ambient temperature. An
aqueous dispersion of biotin-DPPE (1.5 mg) in 4%
propylene glycol-glycerol (1 ml) was added and the
sample was put on a roller table for 1-2 hours. The
suspension was filled into vials and the head spaces
were flushed with perfluorobutane. The vials were
shaken for 45 seconds, whereafter they were put on a
roller table. After centrifugation for 7 minutes the
infranatant was exchanged with water and the washing was
repeated twice. Normal phase HPLC with an Evaporative
Light Scattering Detector confirmed that the membranes
of the microbubbles contained 4 mol% biotin-DPPE. The
mean particle diameter of the microbubbles was 4 μm
measured by Coulter Counter. Ultrasound transmission
measurements using a 3.5 MHz broadband transducer showed

that a particle dispersion of < 2 mg/ml gave a sound
beam attenuation higher than 5 dB/cm.

5 <u>Example 8 - Gas-filled microbubbles encapsulated with
phosphatidylserine and biotinylated antibody non-
covalently bound to streptavidin-Succ-PEG-DSPE</u>

<u>a)   Synthesis of Succ-PEG$_{3400}$-DSPE</u>

10

NH$_2$-PEG$_{3400}$-DSPE (prepared as in Example 2) is
carboxylated using succinic anhydride, e.g. by a similar
method to that described by Nayar, R. and Schroit, A.J.
in *Biochemistry* (1985) **24**, 5967-71.

15

<u>b)   Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine and Succ-PEG$_{3400}$-DSPE</u>

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%)
20   and Succ-PEG$_{3400}$-DSPE (10-0.1 mol%) is added 5% propylene
glycol-glycerol in water (1 ml). The dispersion is
heated to not more than 80 °C for 5 minutes and then
coooled to ambient temperature. The dispersion (0.8 ml)
is transferred to a vial (1 ml) and the head space is
25   flushed with perfluorobutane. The vial is shaken in a
cap-mixer for 45 seconds, whereafter the sample is put
on a roller table. After centrifugation the infranatant
is exchanged with water and the washing is repeated.
Alternatively the microbubbles may be prepared as
30   described in Example 2(f).

<u>c)   Coupling of streptavidin to gas-filled microbubbles
encapsulated with phosphatidylserine and Succ-PEG$_{3400}$-DSPE</u>

35   Streptavidin is covalently bound to Succ-PEG$_{3400}$-DSPE in
the microbubble membranes by standard coupling methods
using a water-soluble carbodiimide. The sample is

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placed on a roller table during the reaction. After
centrifugation the infranatant is exchanged with water
and the washing is repeated. The functionality of the
attached streptavidin is analysed by binding, e.g. to
fluorescently labeled biotin, biotinylated antibodies
(detected with a fluorescently labeled secondary
antibody) or biotinylated and fluorescence- or
radioactively-labeled oligonucleotides. Analysis is
performed by fluorescence microscopy or scintillation
counting.

d) Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine and biotin non-covalently bound
to streptavidin-Succ-PEG$_{3400}$-DSPE

Microbubbles from Example 8(c) are incubated in a
solution containing biotinylated vectors, e.g.
biotinylated antibodies. The vector-coated microbubbles
are washed as described above.

Example 9 - Gas-filled microbubbles encapsulated with
phosphatidylserine and biotinylated oligonucleotide non-
covalently bound to streptavidin-Succ-PEG-DSPE

a) Synthesis of Succ-PEG$_{3400}$-DSPE

NH$_2$-PEG$_{3400}$-DSPE (prepared as in Example 2) is
carboxylated using succinic anhydride, e.g. by a similar
method to that described by Nayar, R. and Schroit, A.J.
in *Biochemistry* (1985) **24,** 5967-71.

b) Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine and Succ-PEG$_{3400}$-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%)
and Succ-PEG$_{3400}$-DSPE (10-0.1 mol%) is added 5% propylene

glycol-glycerol in water (1 ml). The dispersion is
heated to not more than 80 °C for 5 minutes and then
cooled to ambient temperature. The dispersion (0.8 ml)
is transferred to a vial (1 ml) and the head space is
5   flushed with perfluorobutane. The vial is shaken in a
cap-mixer for 45 seconds, whereafter the sample is put
on a roller table. After centrifugation the infranatant
is exchanged with water and the washing is repeated.
Alternatively the microbubbles may be prepared as
10   described in Example 2(f).

c)   Coupling of streptavidin to gas-filled microbubbles
encapsulated with phosphatidylserine and Succ-PEG$_{3400}$-DSPE

15   Streptavidin is covalently bound to Succ-PEG$_{3400}$-DSPE in
the microbubble membraness by standard coupling methods
using a water-soluble carbodiimide. The sample is
placed on a roller table during the reaction. After
centrifugation the infranatant is exchanged with water
20   and the washing is repeated. The functionality of the
attached streptavidin is analyzed by binding, e.g. to
fluorescently labeled biotin, biotinylated antibodies
(detected with a fluorescently labeled secondary
antibody) or biotinylated and fluorescence- or
25   radioactively-labeled oligonucleotides. Analysis is
performed by fluorescence microscopy or scintillation
counting.

d)   Preparation of gas-filled microbubbles encapsulated
30   with phosphatidylserine and a biotinylated
oligonucleotide non-covalently bound to streptavidin-
Succ-PEG$_{3400}$-DSPE

Microbubbles from Example 9(c) are incubated in a
35   solution containing a biotinylated oligonucleotide. The
oligonucleotide-coated bubbles are washed as described
above. Binding of the oligonucleotide to the bubbles is

detected e.g. by using fluorescent-labeled
oligonucleotides for attachment to the bubbles, or by
hybridising the attached oligonucleotide to a labeled
(fluorescence or radioactivity) complementary
5   oligonucleotide. The functionality of the
oligonucleotide-carrying microbubbles is analysed, e.g.
by hybridising the bubbles with immobilized DNA-
containing sequences complementary to the attached
oligonucleotide. As examples, an oligonucleotide
10   complementary to ribosomal DNA (of which there are many
copies per haploid genome) and an oligonucleotide
complementary to an oncogene (e.g. ras of which there is
one copy per haploid genome) may be used.

15

**Example 10 - Gas-filled microbubbles encapsulated with
phosphatidylserine and folate-PEG-Succ-DSPE**

a)   Preparation of folate-PEG-Succ-DSPE

20

Folate-PEG-Succ-DSPE is synthesised as described by Lee,
R.J. and Low, P.S. in (1995) *Biochimica. Biophysica.
Acta* **1233**, 134-144.

25   **b)   Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine and folate-PEG-Succ-DSPE**

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%)
and folate-PEG-DSPE (10-0.1 mol%) is added 5% propylene
30   glycol-glycerol in water (1 ml). The dispersion is
heated to not more than 80 °C for 5 minutes and is then
cooled to ambient temperature. The dispersion (0.8 ml)
is transferred to a vial (1 ml) and the head space is
flushed with perfluorobutane. The vial is shaken in a
35   cap-mixer for 45 seconds, whereafter the sample is put
on a roller table. After centrifugation the infranatant
is exchanged with water and the washing is repeated.

Alternatively the microbubbles are prepared as described
in Example 2(e) or 2(f). Analysis of folate attachment
may for example be done by microscopic study of the
binding of the folate-containing microbubbles to cells
expressing different levels of folate receptors.

Example 11 - Gas-filled microbubbles encapsulated with
phosphatidylserine and thiolated-anti-CD34-Mal-PEG$_{3400}$-
DSPE, thiolated-anti-ICAM-1-Mal-PEG$_{3400}$-DSPE and
thiolated-anti-E-Selectin-Mal-PEG$_{3400}$-DSPE

a) Preparation of thiolated-anti-CD34 antibodies

Thiolation of anti-CD34 antibodies may be effected as
described by Hansen, C.B. *et al.* in (1995) *Biochim.
Biophys. Acta* **1239**, 133-144.

b) Preparation of thiolated-anti-ICAM-1 antibodies

Thiolation of anti-ICAM-1 antibodies may be effected as
described by Hansen, C.B. *et al.* in (1995) *Biochim.
Biophys. Acta* **1239**, 133-144.

c) Preparation of thiolated-anti-E-selectin antibodies

Thiolation of anti-E-selectin antibodies may be effected
as described by Hansen, C.B. *et al.* in (1995) *Biochim.
Biophys. Acta* **1239**, 133-144.

d) Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine and thiolated-anti-CD34-Mal-
PEG$_{3400}$-DSPE, thiolated-anti-ICAM-1-Mal-PEG$_{3400}$-DSPE,
thiolated-anti-E-selectin-Mal-PEG$_{3400}$-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%)
and Mal-PEG$_{3400}$-DSPE (10-0.1 mol%, prepared as in Example

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2) is added 5% propylene glycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and is then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with an appropriate buffer, and coupling of the antibodies from Example 11(a), 11(b) and 11(c) to the microbubbles is performed, e.g. as described by Goundalkar, A., Ghose, T. and Mezei, M. in *J. Pharm. Pharmacol.* (1984) **36**, 465-466 or by Hansen, C.B. *et al.* in (1995) *Biochim. Biophys. Acta* **1239**, 133-144. The microbubbles are placed on a roller table for several hours and are then washed.

Example 12 - The peptide FNFRLKAGQKIRFGAAAWEPPRARI attached to gas-filled microbubbles encapsulated with phosphatidylserine

The peptide FNFRLKAGQKIRFGAAAWEPPRARI, comprising phosphatidylserine-binding and heparin-binding sections, is synthesised. The peptide is added to preformed phosphatidylserine-encapsulated perfluorobutane microbubbles and thoroughly mixed.

Example 13 - Fibronectin covalently bound to gas-filled microbubbles encapsulated with phosphatidylserine and phosphatidylethanolamine

a) Microbubbles preparation

DSPS (25 mg) and DSPE (5.0 mg) were weighed into a clean vial and 5 ml of a solution of 1.4% propylene

glycol/2.4% glycerol was added. The mixture was warmed
to 80°C for 5 minutes. The sample was cooled to room
temperature and the head space was flushed with
perfluorobutane gas. The vials were shaken in a cap
mixer for 45 seconds and the microbubbles were twice
washed with distilled water then resuspended in 0.1 M
sodium borate buffer, pH 9.

b) Modification of fibronectin

Fibronectin (1.0 mg) in 5 ml 0.01 M Hepes buffer, pH 8,
was added to 0.1 mmol of the crosslinker SDBP. The
mixture was incubated on ice for 2 hours.

c) Microbubble modification.

To the protein solution from (b) was added the
microbubble suspension from (a) and incubation was
allowed to proceed for 2 hours at room temperature on a
roller table. Unreacted material was removed by
allowing the microbubbles to float and then replacing
the buffer with 0.1 M sodium borate buffer, pH 9. This
process was repeated three times.

d) In vitro analysis.

The microbubbles were tested in the *in vitro* assay
detailed in Example 21. A gradual accumulation of
microbubbles binding to the cells was observed.

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Example 14 - Gas-filled microbubbles encapsulated with
phosphatidylserine, and 3ß-[N-(N',N'-
dimethylaminoethane)carbamoyl]cholesterol

5   a) Synthesis of 3ß-[N-(N',N'-dimethylaminoethane)-
carbamoyl]cholesterol (DC-chol) (Farhood, H., Gao, X.
Barsoum, J. and Huang, L., Anal. Biochem. 225, 89-93
(1995))

10 To a stirred solution of 2-dimethylaminoethylamine
(19.40 mg, 24:1, 0.22 mmol) and triethylamine (310 µl,
2.23 mmol) in dichloromethane (3 ml) at room temperature
was slowly added a solution of cholesteryl chloroformate
(100 mg, 0.22 mmol) in 1,4-dioxane. When the reaction
15 was completed, the mixture was evaporated to dryness and
the residue was purified by flash chromatography
(CHCl$_3$/MeOH, 4:1). A white solid was obtained, yield 105
mg (95%). The structure was verified by NMR and MALDI.

20 b) Preparation of microbubble dispersion

Monolayer-encapsulated microbubbles containing
perfluorobutane are made from a mixture of 90%
phosphatidylserine and 10% (DC-chol) by weighing DSPS
25 (4.5 mg) and (DC-chol) (0.5 mg) into a 2 ml vial. 0.8
ml propylene glycol/glycerol (4%) in water was added.
The solution was heated at 80°C for 5 minutes and shaken.
The solution was then cooled to ambient temperature and
the headspace was flushed with perfluorobutane. The
30 vial was shaken on a cap-mixer at 4450
oscillations/minute for 45 seconds and put on a roller
table. The sample was washed by centrifuging at 2000
rpm for 5 minutes. The infranatant was removed by a
syringe and distilled water was added to the same
35 volume. The headspace was again flushed with
perfluorobutane and the sample was kept on a roller
table until a homogeneous appearance was obtained. The

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washing procedure was repeated again.

<u>Example 15 - Gas-filled microbubbles encapsulated with</u>
<u>phosphatidylserine and WEPPRARI-PE</u>

Phosphatidylethanolamine (PE) is reacted with an
equimolar amount of the crosslinker *N*-
hydroxysuccinimidyl-2,3-dibromopropionate in a 1:1
mixture of dioxane and 0.02 M HEPES buffer, pH 8.0.
Following incubation for 2 hours on ice, an equimolar
amount of the heparin-binding peptide WEPPRARI is added,
the pH is brought to 9 by the addition of 0.2 M disodium
tetraborate, and the incubation is continued for 2 hours
at room temperature. The reaction product is purified
by chromatography. Monolayer-encapsulated microbubbles
containing perfluorobutane are made from a mixture of
80-95 % phosphatidylserine (PS) and 5-20 % of peptide-
substituted PE.

<u>Example 16 - Gas-filled microbubbles encapsulated with</u>
<u>phosphatidylserine and inactivated human thrombin-Succ-</u>
<u>PEG$_{3400}$-DSPE</u>

<u>a)   Inactivation of human thrombin</u>

Human thrombin was inactivated by incubation with a 20 %
molar excess of D-Phe-L-Pro-L-Arg-chloromethyl ketone in
0.05 M HEPES buffer, pH 8.0, at 37 °C for 30 minutes.

<u>b)   Preparation of gas-filled microbubbles encapsulated</u>
<u>with phosphatidylserine and Succ-PEG$_{3400}$-DSPE</u>

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%)
and Succ-PEG$_{3400}$-DSPE (10-0.1 mol%, prepared as in Example
9(a)) was added 5% propylene glycol-glycerol in water (1

ml). The dispersion was heated to not more than 80 °C
for 5 minutes and was then cooled to ambient
temperature. The dispersion (0.8 ml) was transferred to
a vial (1 ml) and the head space was flushed with
perfluorobutane. The vial was shaken in a cap-mixer for
45 seconds, whereafter the sample was put on a roller
table. After centrifugation the infranatant was
exchanged with water and the washing was repeated.
Alternatively the microbubbles may be prepared as
described in Example 2(f).

c) Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine and inactivated human thrombin-
Succ-PEG$_{3400}$-DSPE

Inactivated human thrombin was covalently bound to Succ-
PEG$_{3400}$-DSPE in the microbubbles from Example 16(b) by
standard coupling methods using a water-soluble
carbodiimide. The sample was placed on a roller table
during the reaction. After centrifugation the
infranatant was exchanged with water and the washing was
repeated.

Example 17 - Gas-filled microbubbles having methotrexate
and prodrug-activating enzyme attached

a) Methotrexate attached via a peptide linker to gas-
filled micrububbles

Methods for attaching aminoacids to the anticancer drug
methotrexate (MTX) are well described in the literature
(see e.g. Huennekens, F.M. (1994), TIBTECH 12, 234-239
and references therein). Instead of a single amino acid
a peptide may be attached to MTX using the same
technology. Such a peptide may constitute a linker for
the attachment of MTX to the surface of microbubbles.

One class of such linkers comprises peptides of the
general structure (MTX)-F-K/R-X-R-Z-C where X is any
amino acid and Z is a hydrophobic amino acid. A
specific example of such a linker is (MTX)-F-K-L-R-L-C.
The SH- group in the Cys-residue is employed for
attachment of the MTX-peptide to the microbubbles (e.g.
composed of phosphatidylserine and Mal-PEG-DSPE) using
standard technology, e.g. as in Example 2. A linker of
this kind is expected to be cleaved by the enzyme
cathepsin B which often is selectively overexpressed
outside and on the surface of tumour cells (Panchal,
R.G. et al. (1996), Nat. Biotechnol. 14, 852-856).
Thus, the potential prodrug (MTX)-F-K/R-X-R would be
liberated selectively in tumours. This prodrug can
further be activated to the active drug MTX by the
action of carboxypeptidases, either present
endogeneously in the tumour or targeted to the tumour
e.g. by tumour-associated antibodies (see below).

b) Prodrug-activating enzyme covalently attached to the
surface of gas-filled microbubbles

An example of a prodrug-activating enzyme is
carboxypeptidase A (CPA), which may be conjugated to the
surface of microbubbles encapsulated by, for example, a
mixture of phosphatidylserine and phosphatidyl-
ethanolamine, e.g. by using a 3400 Da poly(ethylene
glycol) chain bearing an N-hydroxysuccinimide group at
both ends (Perron, M.J. and Page, M., Br. J. Cancer 73,
281-287); the microbubbles may be prepared by standard
methods. Microbubbles containing CPA may be targeted to
areas of pathology by incorporating a suitable targeting
vector in the CPA-containing bubbles. Alternatively CPA
may be attached directly to a vector (e.g. an antibody),
for example by the method as described above. In this
latter case the CPA-vector conjugate will be attached to
the surface of the microbubbles as described in Hansen,

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C.B. et al. (1995) Biochim. Biophys. Acta 1239 133-144.
Examples of the many possible prodrug-enzyme pairs are
described in e.g. Huennekens, F.M. (1994) TIBTECH **12**,
234-239.

Example 18 - Gas-filled microbubbles encapsulated with
phosphatidylserine, thiolated-anti-CEA-Mal-PEG$_{3400}$-DSPE
and the anticancer prodrug 3',5'-O-dipamitoyl-5-fluoro-
2'-deoxyuridine

a)   Preparation of thiolated anti-CEA antibodies

Thiolation of anti-CEA antibodies may be effected as
described by Hansen, C.B. et al. in (1995) Biochim.
Biophys. Acta **1239**, 133-144.

b)   Preparation of gas-filled microbubbles encapsulated
with phosphatidylserine, thiolated-anti-CEA-Mal-PEG$_{3400}$-
DSPE and the anticancer prodrug 3',5'-O-dipamitoyl-5-
fluoro-2'-deoxyuridine

To a mixture (5 mg) of phosphatidylserine (90-99.9
mol%), Mal-PEG$_{3400}$-DSPE (10-0.1 mol%, prepared as in
Example 2) and the anticancer prodrug 3',5'-O-
dipamitoyl-5-fluoro-2'-deoxyuridine (Mori, A. et al.
(1995) Cancer Chemother. Pharmacol. **35**, 447-456) is
added 5% propylene glycol-glycerol in water (1 ml). The
dispersion is heated to not more than 80 °C for 5 minutes
and is then cooled to ambient temperature. The
dispersion (0.8 ml) is transferred to a vial (1 ml) and
the head space is flushed with perfluorobutane. The
vial is shaken in a cap-mixer for 45 seconds, whereafter
the sample is put on a roller table. After
centrifugation the infranatant is exchanged with an
approperiate buffer, and coupling of the antibody to the
microbubble is performed, e.g. as described by

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Goundalkar, A., Ghose, T. and Mezei, M. in *J. Pharm. Pharmacol.* (1984) **36** 465-466 or by Hansen, C.B. *et al.* in (1995) *Biochim. Biophys. Acta* **1239** 133-144. The microbubbles are placed on a roller table for several hours and are then washed.

Example 19 - Gas-filled microbubbles encapsulated with phosphatidylserine, thiolated-anti-CEA-Mal-PEG$_{3400}$-DSPE and the anticancer prodrug N-trifluoroacetyl-adriamycin-14-valerate

a) Preparation of thiolated anti-CEA antibodies

Thiolation of anti-CEA antibodies may be effected as described by Hansen, C.B. *et al.* in (1995) *Biochim. Biophys. Acta* **1239** 133-144.

b) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine, thiolated-anti-CEA-Mal-PEG$_{3400}$-DSPE and the anticancer prodrug N-trifluoroacetyl-adriamycin-14-valerate

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%), Mal-PEG$_{3400}$-DSPE (10-0.1 mol%, prepared as in Example 2) and the anticancer prodrug N-trifluoroacetyl-adriamycin-14-valerate (Mori, A. *et al.* (1993) *Pharm. Res.* **10**, 507-514), is added 5% propylene glycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and is then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with an appropriate buffer, and coupling of the antibody to the microbubble is performed, e.g. as

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described by Goundalkar, A., Ghose, T. and Mezei, M. in
J. Pharm. Pharmacol. (1984) **36** 465-66 or by Hansen, C.B.
et al. in (1995) *Biochim. Biophys. Acta* **1239** 133-144.
The microbubbles are placed on a roller table for
several hours and are then washed.

Example 20 - Method of use

An agent comprising phosphatidylserine-encapsulated
microbubbles having inactivated human thrombin-Succ-
PEG_{3400}-DSPE incorporated into the encapsulating membrane
is lyophilised from 0.01 M phosphate buffer, pH 7.4.
The product is redispersed in sterile water and injected
intravenously into a patient with suspected venous
thrombosis in a leg vein. The leg is examined by
standard ultrasound techniques. The thrombus is located
by increased contrast as compared with surrounding
tissue.

Example 21 - Preparation and biological evaluation of gas-containing microbubbles of DSPS `doped' with a lipopeptide comprising a heparin sulphate binding peptide (KRKR) and a fibronectin peptide (WOPPRARI)

This example is directed at the preparation of targeted
microbubbles comprising multiple peptidic vectors
arranged in a linear sequence.

a) Synthesis of a lipopeptide consisting of a heparin sulphate binding peptide (KRKR) and fibronectin peptide (WOPPRARI)

The lipopeptide was synthesised on an ABI 433A automatic peptide synthesiser starting with Fmoc-Ile-Wang resin on a 0.1 mmol scale using 1 mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU before coupling. The simultaneous removal of peptide from the resin and side-chain protecting groups was carried out in TFA containing 5% phenol, 5% EDT, 5% anisole and 5% H_2O for 2 hours, giving a crude product yield of 150 mg. Purification by preparative HPLC of a 40 mg aliquot of crude material was carried out using a gradient of 70 to 100% B over 40 minutes (A = 0.1% TFA/water and B = MeOH) at a flow rate of 9 ml/min. After lyophilisation, 16 mg of pure material were obtained (analytical HPLC, gradient 70-100% B where B = MeOH, A = 0.01% TFA/water: detection - UV 260 and fluorescence, Ex_{280}, Em_{350} - product retention time = 19.44 minutes). Further product characterisation was carried out using MALDI mass spectrometry: expected M+H at 2198, found at 2199.

b) <u>Preparation of gas-filled microbubbles of DSPS
'doped' with a multiple-specific lipopeptide consisting
of a heparin sulphate binding peptide (KRKR) and
fibronectin peptide (WQPPRARI)</u>

DSPS (4.5 mg) and lipopeptide from (a) (0.5 mg) were
weighed into each of two vials and 0.8 ml of a solution
of 1.4% propylene glycol/2.4% glycerol was added to each
vial. The mixtures were warmed to 80°C for 5 minutes
(vials shaken during warming). The samples were cooled
to room temperature and the head spaces flushed with
perfluorobutane gas. The vials were shaken in a cap
mixer for 45 seconds and rolled overnight. The
resulting microbubbles were washed several times with
deionised water and analysed by Coulter counter [size:
1-3 micron (87%), 3-5 micron (11.5%)] and acoustic
attenuation (frequency at maximum attenuation: 3.5 MHz).
The microbubbles were stable at 120 mm Hg. MALDI mass
spectral analysis was used to confirm incorporation of
lipopeptide into DSPS microbubbles as follows: ca. 0.05-
0.1 ml of microbubble suspension was transferred to a
clean vial and 0.05-0.1 ml methanol was added. The
suspension was sonicated for 30 seconds and the solution
was analysed by MALDI MS. Positive mode gave M+H at
2200 (expected for lipopeptide, 2198).

c) <u><i>In vitro</i> study of gas-filled microbubbles of DSPS
'doped' with a multiple-specific lipopeptide consisting
of a heparin sulphate-binding peptide (KRKR) and
fibronectin peptide (WQPPRARI): binding to endothelial
cells under flow conditions</u>

The human endothelial cell line ECV 304, derived from a
normal umbilical cord (ATCC CRL-1998) was cultured in
260 mL Nunc culture flasks (chutney 153732) in RPMI 1640
medium to which L-glutamine (200 mM), penicillin/
streptomycin (10,000 U/ml and 10,000 µg/ml) and 10%

fetal bovine serum were added. The cells were
subcultured with a split ratio of 1:5 to 1:7 when
reaching confluence. Cover-glasses, 22 mm in diameter,
were sterilised and placed on the bottom of 12 well
culture plates, whereafter cells in 0.5 ml complete
medium with serum were added above the plates. When the
cells reached confluence the coverslips were placed in a
custom-made flow chamber consisting of a groove carved
into a glass plate upon which the cover slip with cells
was placed, with the cells facing the groove, so as to
form a flow channel. Microbubbles prepared as in (b)
were passed from a reservoir held at 37 °C through the
flow chamber and back to the reservoir using a
peristaltic pump. The flow rate was adjusted to
simulate physiologically relevant shear rates. The flow
chamber was placed under a microscope and the
interaction between the microbubbles and cells was
viewed directly. A camera mounted on the microscope was
connected to a colour video printer and a monitor. A
gradual accumulation of microbubbles on the cells took
place at a rate dependent on the flow rate. On further
increasing the flow rate, cells started to become
detached from the coverslip, but the microbubbles
remained bound to the cells. Control bubbles not
carrying the vector did not adhere to the endothelial
cells and disappeared from the chamber under minimal
flow conditions.

d) *In vivo experiment in dog*

Case 1)

A 22 kg mongrel dog was anaesthetised with pentobarbital
and mechanically ventilated. The chest was opened by a
midline sternotomy, the anterior pericardium was
removed, and a 30 mm gelled silicone rubber spacer was
inserted between the heart and a P5-3 transducer of an

ATL HDI-3000 ultrasound scanner. The scanner was set
for intermittent short axis imaging once in each
end-systole by delayed EGC triggering. A net volume of
2 ml of microbubbles from (b) was injected as a rapid

5   intravenous bolus; 3 seconds later, the imaged right
ventricle was seen to contain contrast material, and
another 3 seconds later the left ventricle was also
filled and a transient attenuation shadow which obscured
the view of the posterior parts of the left ventricle

10   was observed. Substantial increases in brightness were
seen in the myocardium and, when the attenuation shadow
subsided, in the portions of the heart distal to the
left ventricle. After passage of the inital bolus, the
ultrasound scanner was set to continuous, high frame

15   rate, high output power imaging, a procedure known to
cause destruction of ultrasound contrast agent
microbubbles in the imaged tissue regions. After a few
seconds, the scanner was adjusted back to its initial
setting. The myocardium was then darker, and closer to

20   the baseline value. Moving the imaged slice to a new
position resulted in re-appearance of contrast effects;
moving the slice back to the initial position again
resulted in a tissue brightness close to baseline.

25   Case 2) [comparative]

A net volume of 2 ml microbubbles prepared in an
identical manner to (b) above with the exception that no
lipopeptide was included in the preparation was

30   injected, using the same imaging procedure as above.
The myocardial echo enhancement was far less intense and
of shorter duration than that observed in Case 1. At
the completion of the left ventricular attenuation
phase, there was also almost complete loss of myocardial

35   contrast effects, and the myocardial echo increases in
the posterior part of the left ventricle noted in Case 1
were not observed.

Example 22 - Preparation of gas-filled microbubbles
encapsuled with DSPS comprising thiolated anti-CD34-MAL-
PEG $_{2000}$-PE

a) Preparation of gas-filled microbubbles encapsuled
with DSPS and PE-PEG $_{2000}$-Mal

DSPS (4.5 mg, 3.9 mmol) and PE-PEG$_{2000}$-Mal from Example 50
(0.5 mg) were weighed into a clean vial and 1 ml of a
solution of 1.4% propylene glycol/2.4% glycerol was
added. The mixture was warmed to 80°C for 5 minutes
then filtered through a 4.5 micron filter. The sample
was cooled to room temperature and the head space was
flushed with perfluorbutane gas. The vials were shaken
in a cap mixer for 45 seconds and the resulting
microbubbles were washed three times with distilled
water.

b) Thiolation of anti-CD34 antibodies

To 0.3 mg of anti-CD34 antibody dissolved in 0.5 ml
phosphate buffered saline (PBS), pH7, was added 0.3 mg
Traut's reagent and the solution was stirred at room
temperature for 1 hour. Excess reagent was separated
from the modified protein on a NAP-5 column.

c) Conjugation of thiolated anti-CD34 antibody to gas-
filled microbubbles encapsuled with DSPS and comprising
DSPE-PEG $_{2000}$-MAL

0.5 ml of the thiolated antibody praparation from (b)
was added to an aliquot of microbubbles from (a) and the
conjugation reaction was allowed to proceed for 30
minutes on a roller table. Following centifugation at
2000 rpm for 5 minutes the infranatant was removed. The
microbubbles were washed a further three times with
water.

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d) Detection of the antibody encapsulated in the
microbubbles using a FITC-conjugated secondary antibody

To the microbubble suspension from (c) was added 0.025
mL FITC-conjugated goat-anti-mouse antibody. The
mixture was incubated in the dark at room temperature
for 30 minutes on a roller table and was then
centrifuged at 2000 rpm for 5 minutes. The infranatant
was then removed and the microbubbles were washed a
further three times with water. Flow cytometric
analysis of the microbubble suspension showed that 98%
of the population was fluorescent.

Example 23 - Preparation of gas-filled microbubbles
encapsuled with DSPS comprising thiolated anti-CD62-MAL-
PEG $_{2000}$-PE

An identical procedure to that described in Example 22
was used to prepare microbubbles comprising anti-CD62
antibodies.

Example 24 - Preparation of gas-filled microbubbles
encapsuled with DSPS comprising thiolated anti-ICAM-1-
MAL-PEG $_{2000}$-PE

An identical procedure to that described in Example 22
was used to prepare microbubbles comprising anti-ICAM-1
antibodies.

Example 25 - Preparation of gas-filled microbubbles
encapsulated with DSPS and thiolated anti-CD62-Mal-
PEG$_{2000}$-PE and thiolated-anti-ICAM-1-Mal-PEG$_{2000}$-PE

5   This example is directed to the preparation of
microbubbles comprising multiple antibody vectors for
targeted ultrasound imaging.

a) Preparation of gas-filled microbubbles encapsulated
10 with DSPS and PE-PEG$_{2000}$-Mal

DSPS (4.5 mg) and PE-PEG$_{2000}$-Mal from Example 2 (a) (0.5
mg) were weighed into a clean vial and 1 ml of a
solution of 1.4% propylene glycol/2.4% glycerol was
15 added. The mixture was warmed to 80°C for 5 minutes and
then filtered through a 4.5 micron filter. The sample
was cooled to room temperature and the head space was
flushed with perfluorobutane gas. The vials were shaken
in a cap mixer for 45 seconds and the microbubbles were
20 washed three times with distilled water.

b) Thiolation of anti-CD62 and anti-ICAM-1 antibodies

To 0.3 mg each of anti-CD62 and anti-ICAM-1 antibodies
25 dissolved in PBS buffer (pH 7, 0.5 ml) was added Traut's
reagent and the solutions were stirred at room
temperature for 1 hour. Excess reagent was separated
from the modified protein on a NAP-5 column.

30 c) Conjugation of thiolated anti-CD62 and anti-ICAM-1
antibodies to gas-filled microbubbles encapsulated with
DSPS and DSPE-PEG$_{2000}$-Mal

0.5 ml of the mixed thiolated antibody preparation from
35 (b) was added to an aliquot of microbubbles from (a) and
the conjugation reaction was allowed to proceed for 30
minutes on a roller table. Following centrifugation at

2000 rpm for 5 minutes, the infranatant was removed.
The microbubbles were washed a further three times with
water.

5   The PEG spacer length may be varied to include longer
(e.g. PEG_{3400} and PEG_{5000}) or shorter (e.g. PEG_{600} or PEG_{800})
chains. Addition of a third antibody such as thiolated-
anti-CD34 is also possible.

10

Example 26 - Targeted gas-filled microbubbles comprising
DSPS coated non-covalently with polylysine and a fusion
peptide comprising a PS-binding component and a
fibronectin peptide sequence FNFRLKAGQKIRFGGGGWQPPRAI

15

a) Synthesis of PS-binding/fibronectin fragment fusion
peptide FNFRLKAGQKIRFGGGGWQPPRAI

The peptide was synthesised on an ABI 433A automatic
20   peptide synthesiser starting with Fmoc-Ile-Wang resin on
a 0.1 mmol scale using 1 mmol amino acid cartridges.
All amino acids were preactivated using HBTU before
coupling. The simultaneous removal of peptide from the
resin and side-chain protecting groups was carried out
25   in TFA containing 5% phenol, 5% EDT and 5% H_2O for 2
hours, giving a crude product yield of 302 mg.
Purification by preparative HPLC of a 25 mg aliquot of
crude material was carried out using a gradient of 20 to
40% B over 40 minutes (A = 0.1% TFA/water and B = 0.1%
30   TFA/acetonitrile) at a flow rate of 9 ml/min. After
lyophilisation 10 mg of pure material was obtained
(analytical HPLC, gradient 20 to 50% B where B = 0.1%
TFA/acetonitrile, A = 0.01% TFA/water: detection - UV
214 and 260 nm - product retention time = 12.4 minutes).
35   Further product characterization was carried out using
MALDI mass spectrometry: expected M+H at 2856, found at
2866.

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b)   Preparation of gas-filled microbubbles comprising
DSPS coated non-covalently with polylysine and the PS-
binding/fibronectin fragment fusion peptide
FNFRLKAGOKIRFGGGGWOPPRAI

DSPS (5 mg) was weighed into a clean vial along with
poly-L-lysine (0.2 mg) and peptide from (a) above (0.2
mg). To the vial was added 1.0 ml of a solution of 1.4%
propylene glycol/2.4% glycerol. The mixture was warmed
to 80°C for 5 minutes. The sample was cooled to room
temperature and the head space was flushed with
perfluorobutane gas. The vials were shaken in a cap
mixer for 45 seconds and the resulting microbubbles were
centrifuged at 1000 rpm for 3 minutes. Following
extensive washing with water, PBS and water, the final
solution was examined for polylysine and peptide content
using MALDI MS. No polypeptide material was observed in
the final wash solution. Acetonitrile (0.5 ml) was then
added and the microbubbles were destroyed by sonication.
Analysis of the resulting solution for polylysine and
PS-binding/fibronectin fusion peptide was then carried
out using MALDI MS. The results were as follows:

	MALDI expected	MALDI found
Poly-L-lysine	786, 914,	790, 919,
	1042, 1170	1048, 1177
DSPS-binding peptide	2856	2866

The spacer element contained within the PS-
binding/fibronectin fusion peptide (-GGG-) may also be
replaced with other spacers such as PEG_{2000} or poly
alanine (-AAA-). A form of pre-targeting may also be
employed, whereby the DSPS-binding/fibronectin fragment
fusion peptide is firstly allowed to associate with
cells via fibronectin peptide binding, followed by
administration of PS microbubbles which then bind to the
PS-binding peptide.

Example 27 - Gas-filled microbubbles encapsulated with
phosphatidylserine and biotin-PEG$_{3400}$-alanyl-cholesterol
and functionalised with streptavidin/biotinyl-
endothelin-1 peptide (biotin-D-Trp-Leu-Asp-Ile-Ile-
Trp.OH) and biotinyl-fibrin-anti-polymerant peptide
(biotin-GPRPPERHQS.NH$_2$)

This example is directed at the preparation of targeted
ultrasound microbubbles whereby streptavidin is used as
a linker between biotinylated reporter(s) and vector(s).

a)   Synthesis of biotin-PEG$_{3400}$-b-Alanine cholesterol

To a solution of cholesteryl-b-alanine hydrochloride (as
described in Example 59) (15 mg, 0.03 mmol) in 3 ml
chloroform/wet methanol (2.6:1) was added triethylamine
(42 ml, 0.30 mmol). The mixture was stired for 10
minutes at room temperature and a solution of biotin-
PEG$_{3400}$-NHS (100 mg, 0.03 mmol) in 1,4-dioxane (1 ml) was
added dropwise. After stirring at room temperature for
3 hours, the mixture was evaporated to dryness and the
residue purified by flash chromatography to give white
crystals, yield 102 mg (89%). The structure was
verified by MALDI-MS and NMR.

b)   Synthesis of biotinylated endothelin-1 peptide
(biotin-D-Trp-Leu-Asp-Ile-Ile-Trp.OH)

The peptide was synthesised on a ABI 433A automatic
peptide synthesiser starting with Fmoc-Trp(Boc)-Wang
resin on a 0.1 mmol scale using 1 mmol amino acid
cartridges. All amino acids were preactivated using
HBTU before coupling. The simultaneous removal of
peptide from the resin and side-chain protecting groups
was carried out in TFA containing 5% anisole and 5% H$_2$O
for 2 hours giving a crude product yield of 75 mg.
Purification by preparative HPLC of a 20 mg aliquot of

crude material was carried out using a gradient of 30 to
80% B over 40 minutes (A = 0.1% TFA/water and B = 0.1%
TFA/acetonitrile) and a flow rate of 9 ml/min. After
lyophilisation of the pure fractions 2 mg of pure

5   material was obtained (analytical HPLC, gradient 30-80 %
B where B = 0.1% TFA/acetonitrile, A = 0.01% TFA/water:
detection - UV 214 nm - product retention time = 12.6
minutes). Further product characterization was carried
out using MALDI mass spectrometry: expected M+H at 1077,

10   found at 1077.

c) Synthesis of biotinyl-fibrin-anti-polymerant peptide
(biotin-GPRPPERHQS.NH₂)

15   This peptide was synthesised and purified using similar
protocols to those described in (b) above. The pure
product was characterised by HPLC and MALDI MS.

d) Preparation of multiple-specific gas-filled
20   microbubbles encapsulated with phosphatidylserine and
biotin-PEG₃₄₀₀-b-Alanine cholesterol

DSPS (4.5 mg) and biotin-PEG₃₄₀₀-b-alanine cholesterol
from (a) (0.5 mg) were weighed into a vial and 0.8 ml of

25   a solution of 1.4% propylene glycol/2.4% glycerol was
added. The mixture was warmed to 80°C for 5 minutes
(vials shaken during warming). The sample was cooled to
room temperature and the head space was flushed with
perfluorobutane gas. The vial was shaken in a cap-mixer

30   for 45 seconds and the vial was rolled overnight. The
microbubble suspension was washed several times with
deionised water and analysed by Coulter counter and
acoustic attenuation.

35   e) Conjugation with fluorescein-labelled streptavidin
and biotinylated peptides from (b) and (c)

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To the microbubble preparation from (d) was added
fluorescein-conjugated streptavidin (0.2 mg) dissolved
in PBS (1 ml). The bubbles were placed on a roller
table for 3 hours at room temperature. Following
5   extensive washing with water and analysis by
fluorescence microscopy, the microbubbles were incubated
in 1 ml of PBS containing biotinyl-endothelin-1 peptide
(0.5 mg) and biotinyl-fibrin-anti-polymerant peptide
(0.5 mg) from (b) and (c) respectively for 2 hours.
10   Extensive washing of the microbubbles was performed to
remove unconjugated peptide.

Example 28 - Gas-filled microbubbles encapsulated with
15   phosphatidylserine and biotin-DPPE used to prepare a
streptavidin `sandwich' with a mixture of biotinyl-
endothelin-1 peptide (biotin-D-Trp-Leu-Asp-Ile-Ile-
Trp.OH) and biotinyl-fibrin-anti-polymerant peptide
(biotin-GPRPPERHQS.NH₂)

20

a) Preparation of biotin-containing microbubbles

To a mixture of phosphatidylserine (5 mg) and biotin-
DPPE (0.6 mg) in a clean vial was added 5% propylene
25   glycol-glycerol in water (1 ml). The dispersion was
heated to 80 °C for 5 minutes and then cooled to ambient
temperature. The head space was then flushed with
perfluorobutane and the vial was shaken in a cap-mixer
for 45 seconds. After centrifugation the infranatant
30   was removed and the microbubbles were washed extensively
with water.

b)   Conjugation of gas-filled microbubbles encapsulated
with phosphatidylserine and biotin-DPPE with
streptavidin and a mixture of biotinyl-endothelin-1
(biotin-D-Trp-Leu-Asp-Ile-Ile-Trp.OH) and biotinyl-
fibrin-anti-polymerant peptide (biotin-GPRPPERHOS.NH₂)

The procedure detailed in Example 27 was followed.

Example 29 - PFB gas-containing microbubbles of DSPS
functionalised with Heparin sulphate binding
peptide/Fibronectin peptide/RGD peptide and fluorescein.

a)   Synthesis of a lipopeptide containing the RGD
sequence and a fluorescein reporter group: Dipalmitoyl-
Lys-Lys-Lys-Lys[acetyl-Arg-Gly-Asp-
Lys(fluorescein)]Gly.OH
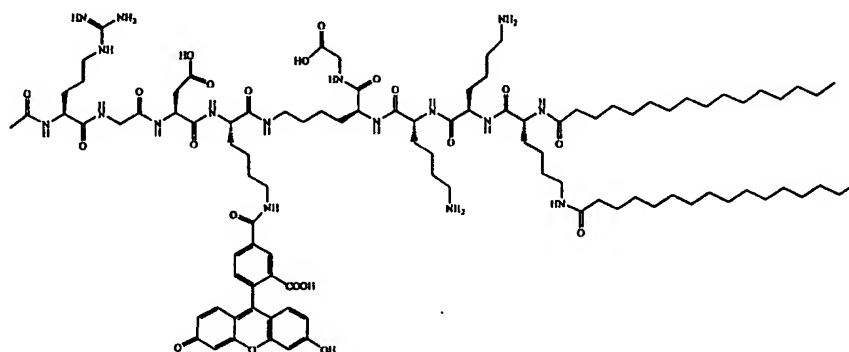
The lipopeptide was synthesised as described in Example
21(a) using commercially available amino acids and
polymers. The lipopeptide was cleaved from the resin in
TFA containing 5% water, 5% phenol and 5% EDT for 2
hours. Following evaporation *in vacuo* the crude product
was precipitated and triturated with diethyl ether.
Purification by preparative HPLC of a 40 mg aliquot of
crude material was carried out using a gradient of 60 to
100% B over 40 minutes (A = 0.1% TFA/water and B = 0.1%

TFA/acetonitrile) at a flow rate of 9 ml/min. After
lyophilisation 10 mg of pure material (analytical HPLC,
gradient 60-100% B where B = 0.1% TFA/acetonitrile, A =
0.01% TFA/water: detection - UV 260 -product retention

5   time = 20-22 minutes). Further product characterisation
was carried out using MALDI mass spectrometry: expected
M+H at 1922, found at 1920.

b)   Synthesis of a lipopeptide containing a heparin
10   sulphate-binding sequence and a fibronectin peptide

Synthesis and purification were carried out as described
in Example 21 (a).

15   c)   Preparation of multiple-specific gas-filled
microbubbles of DSPS functionalised with a heparin
sulphate-binding peptide, a fibronectin peptide, acetyl-
RGD peptide and fluorescein

20   DSPS (4 mg, 3.9 mmol), lipopeptide from (a) (0.5 mg, 0.2
mmol) and lipopeptide from (b) (0.5 mg) were weighed
into each of two vials and 0.8 ml of a solution of 1.4%
propylene glycol/2.4% glycerol was added to each vial.
The mixtures were warmed to 80°C for 5 minutes (vials

25   shaken during warming). The samples were cooled to room
temperature and the head spaces were flushed with
perfluorobutane gas. The vials were shaken in a cap
mixer for 45 seconds and then rolled overnight. The
microbubbles so obtained were washed several times with

30   deionised water and analysed by MALDI mass spectrometry
as described in Example 21(b). The microbubbles were
investigated by microscopy and were seen to have a range
of sizes between 1 and 5 microns. Furthermore the
microbubbles were fluorescent.

35

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Example 30 - Gas-filled microbubbles comprising DSPS
covalently modified with CD71 FITC-labelled anti-
transferrin receptor antibody and `doped' with a
lipopeptide with affinity for endothelial cells

This example is directed at the preparation of multiple
vector targeted ultrasound agents.

a)   Synthesis of an endothelial cell binding
lipopeptide: 2-n-hexadecylstearyl-Lys-Leu-Ala-Leu-Lys-
Leu-Ala-Leu-Lys-Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala-NH₂

The lipopeptide shown below was synthesised on a ABI
433A automatic peptide synthesiser starting with a Rink
amide resin on a 0.1 mmol scale using 1 mmol amino acid
cartridges.
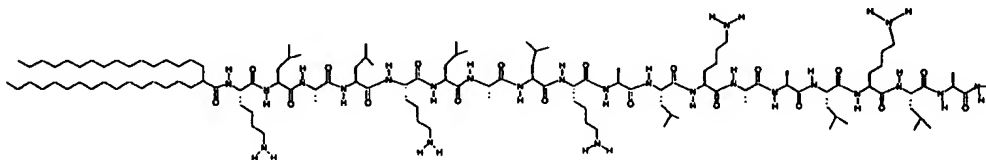
All amino acids and 2-n-hexadecylstearic acid were
preactivated using HBTU before coupling. The
simultaneous removal of peptide from the resin and side-
chain protecting groups was carried out in TFA
containing 5% EDT and 5% H₂O for 2 hours, giving a crude
product yield of 150 mg. Purification by preparative
HPLC of a 40 mg aliquot of crude material was carried
out using a gradient of 90 to 100% B over 50 minutes (A
= 0.1% TFA/water and B = MeOH) at a flow rate of 9
ml/min. After lyophilisation, 10 mg of pure material
was obtained (analytical HPLC, gradient 90-100% B where
B = MeOH, A = 0.01% TFA/water: detection - UV 214 nm -
product retention time = 23 minutes). Further product
characterisation was carried out using MALDI mass
spectrometry: expected M+H at 2369, found at 2373.

b) __Preparation of gas-filled microbubbles comprising__
__DSPS `doped' with a endothelial cell-binding lipopeptide__
__and PE-PEG$_{2000}$-Mal__

5   DSPS (4.5 mg) and lipopeptide from (a) (0.5 mg) along
       with PE-PEG$_{2000}$-Mal from Example 50 (0.5 mg) were weighed
       into a clean vial and 1 ml of a solution of 1.4%
       propylene glycol/2.4% glycerol was added. The mixture
       was warmed to 80°C for 5 minutes and then filtered

10   through a 4.5 micron filter. The sample was cooled to
       room temperature and the head space was flushed with
       perfluorobutane gas. The vial was shaken in a cap mixer
       for 45 seconds and the resulting microbubbles were
       washed three times with distilled water.

15

       c) __Thiolation of FITC-labelled anti-transferrin__
       __receptor antibody__

       FITC-labelled CD71 anti-transferrin receptor Ab (100
20   mg/ml in PBS, 0.7 ml) was reacted with Traut's reagent
       (0.9 mg) at room temperature for 1 hour. Excess reagent
       was separated from modified protein on a NAP-5 column.

       d) __Conjugation of thiolated FITC-labelled anti-__
25   __transferrin receptor antibody to gas-filled microbubbles__
       __comprising DSPS `doped' with an endothelial cell-binding__
       __lipopeptide and DSPE-PEG$_{2000}$-Mal__

       A 0.5 ml aliquot of the protein fraction (2 ml in total)
30   from (c) above was added to the microbubbles from (b)
       and the conjugation reaction was allowed to proceed for
       10 minutes on a roller table. Following centrifugation
       at 1000 rpm for 3 minutes the protein solution was
       removed and the conjugation repeated twice more with 1
35   ml and 0.5 ml aliquots of protein solution respectively.
       The bubbles were then washed four times in distilled
       water and a sample analysed for the presence of antibody

by flow cytometry and microscopy. A fluorescent
population of >92% was observed.

Fig. 1 of the accompanying drawings represents the flow
5   cytometric comparison of negative control microbubbles
of DSPS (left curve) with bubbles conjugated with CD71
FITC-labelled anti-transferrin antibody (filled curve,
right), showing that 92% of the population fluoresce.

10   Incorporation of lipopeptide into the microbubbles was
confirmed by MALDI mass spectrometry as described in
Example 21 (b).

15   Example 31 - Gas-filled microbubbles comprising DSPS, a
lipopeptide for endothelial cell targeting and a
captopril-containing molecule

This example is directed to the preparation of
20   ultrasound agents for combined targeting and therapeutic
applications.

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a)   Synthesis of a lipopeptide functionalised with captopril

5

10
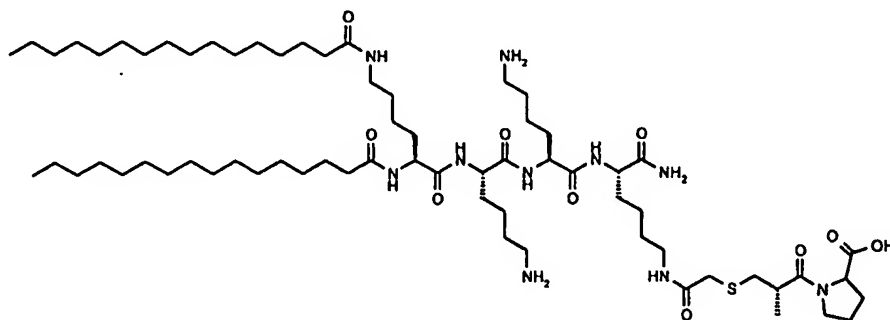
The structure shown above was synthesised using a manual
nitrogen bubbler apparatus starting with Fmoc-protected
Rink Amide MBHA resin on a 0.125 mmol scale. Coupling
15   was carried out using standard TBTU/HOBt/DIEA protocols.
Bromoacetic acid was coupled through the side-chain of
Lys as a symmetrical anhydride using DIC preactivation.
Captopril dissolved in DMF was introduced on the solid-
phase using DBU as base. Simultaneous removal of the
20   peptide from the resin and deprotection of side-chain
protecting groups was carried out in TFA containing 5%
EDT, 5% water and 5% ethyl methyl sulphide for 2 hours.
An aliquot of 10 mg of the crude material was purified
by preparative liquid chromatography using a gradient of
25   70 to 100% B over 60 minutes (A = 0.1% TFA/water and B =
0.1% TFA/acetonitrile) at a flow rate of 10 ml/min.
After lyophilisation a yield of 2 mg of pure material
was obtained (analytical HPLC, gradient 70-100% B over
20 minutes, A = 0.1% TFA/water and B = 0.1%
30   TFA/acetonitrile, flow rate 1 ml/min., detection UV 214
nm, retention time 26 minutes). Further
characterisation was carried out using MALDI mass
spectrometry, giving M+H at 1265 as expected.

35

b)   Synthesis of a lipopeptide with affinity for
endothelial cells: Dipalmitoyl-Lys-Lys-Lys-Aca-Ile-Arg-
Arg-Val-Ala-Arg-Pro-Pro-Leu-NH₂
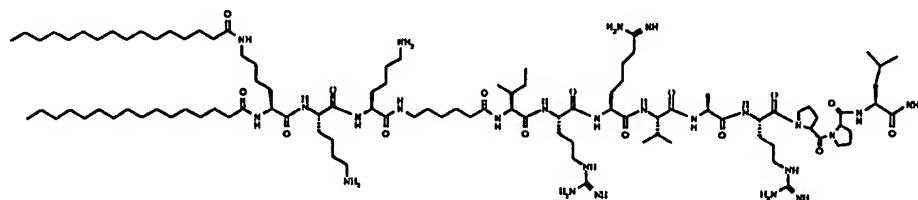
The lipopeptide was synthesised on a ABI 433A automatic
peptide synthesiser starting with Rink amide resin on a
0.1 mmol scale using 1 mmol amino acid cartridges.   All
amino acids and palmitic acid were preactivated using
HBTU before coupling.   The simultaneous removal of
peptide from the resin and side-chain protecting groups
was carried out in TFA containing 5% phenol, 5% EDT and
5% H_2O for 2 hours, giving a crude product yield of 160
mg.   Purification by preparative HPLC of a 35 mg aliquot
of crude material was carried out using a gradient of 70
to 100% B over 40 minutes (A = 0.1% TFA/water and B =
MeOH) at a flow rate of 9 ml/min.   After lyophilisation,
20 mg of pure material was obtained (analytical HPLC,
gradient 70-100% B where B = MeOH, A = 0.01% TFA/water:
detection - UV 214 and 260 nm - product retention time =
16 minutes).   Further product characterisation was
carried out using MALDI mass spectrometry: expected M+H
at 2050, found at 2055.

c)   Preparation of gas-filled microbubbles comprising
DSPS, a lipopeptide for endothelial cell targeting and a
captopril-containing molecule for drug delivery

DSPS (4.5 mg), product from (a) (0.5 mg) and product
from (b) (0.5 mg) were weighed into a vial and 1.0 ml of
a solution of 1.4% propylene glycol/2.4% glycerol was

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added.   The mixture was warmed to 80°C for 5 minutes
(vial shaken during warming). The sample was cooled to
room temperature and the head space was flushed with
perfluorobutane gas. The vial was firstly shaken in a
cap-mixer for 45 seconds then rolled for 1 hour,
whereafter the contents were extensively washed with
deionised water. No detectable level of starting
material was found in the final wash solution as
evidenced by MALDI MS. MALDI mass spectral analysis was
used to confirm incorporation of the products from (a)
and (b) into the microbubbles as described in Example
21(b).

d) *In vitro* study of gas-filled microbubbles comprising
DSPS, a lipopepitde for endothelial cell targeting and a
captopril-containing molecule for therapeutic
applications

The *in vitro* assay decribed in Example 21(c) was used to
examine cell binding under flow conditions. A gradual
accumulation of microbubbles on the cells took place,
depending on the flow rate. On further increasing the
flow rate cells started to become detached from the
coverslip, but the microbubbles remained bound to the
cells. Control microbubbles not carrying the vector did
not adhere to the endothelial cells and disappeared from
the chamber under minimal flow conditions.

Example 32 - Preparation of gas-filled microbubbles
comprising DSPS loaded with a lipopeptide comprising a
helical peptide with affinity for cell membranes and the
peptide antibiotic polymixin B sulphate

This example is directed to the preparation of targeted
microbubbles comprising multiple peptidic vectors having
a combined targeting and therapeutic application.

a)   Synthesis of a lipopeptide comprising a helical
peptide with affinity for cell membranes:
hexadecylstearyl-Lys-Leu-Ala-Leu-Lys-Leu-Ala-Leu-Lys-
Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala-NH$_2$

This is prepared as described in Example 30(a).

b)   Preparation of multiple-specific gas-filled
microbubbles

DSPS (5.0 mg), lipopeptide from (a)(0.3 mg) and
polymixin B sulphate (0.5 mg) were weighed into a clean
vial and 1.0 ml of a solution of 1.4% propylene glycol/
2.4% glycerol was added. The mixture was sonicated for
3-5 minutes, warmed to 80°C for 5 minutes and then
filtered through a 4.5 micron filter. The mixture was
cooled to room temperature and the head space was
flushed with perfluorobutane gas. The vial was shaken
in a cap-mixer for 45 seconds and the resulting
microbubbles were centrifuged at 1000 rpm for 3 minutes.
The microbubbles were washed with water until no
polymixin B sulphate or lipopeptide could be detected in
the infranatant by MALDI-MS. Microscopy showed that the
size distribution of the bubble population was in the
desired range of 1-8 micron. To the washed bubbles (ca.
0.2 ml) was added methanol (0.5 ml), and the mixture was
placed in a sonicator bath for 2 minutes. The resulting
clear solution, on analysis by MALDI-MS, was found to
contain both lipopeptide and polymixin B sulphate
(expected 1203, found 1207).

<u>Example 33 - Preparation of gas-filled microbubbles</u>
<u>comprising DSPS `doped' with a lipopeptide comprising a</u>
<u>IL-1 receptor-binding sequence and modified with a</u>
<u>branched structure containing the drug methotrexate</u>

This example is directed to the preparation of targeted
microbubbles comprising multiple vectors for
targeted/therapeutic applications.

<u>a)   Synthesis of a lipopeptide comprising an</u>
<u>interleukin-1 receptor-binding peptide: Dipalmitoyl-Lys-</u>
<u>Gly-Asp-Trp-Asp-Gln-Phe-Gly-Leu-Trp-Arg-Gly-Ala-Ala.OH</u>

The lipopeptide was synthesised on a ABI 433A automatic
peptide synthesiser starting with Fmoc-Ala-Wang resin on
a 0.1 mmol scale using 1 mmol amino acid cartridges.
All amino acids and palmitic acid were preactivated
using HBTU before coupling. The simultaneous removal of
lipopeptide from the resin and side-chain protecting
groups was carried out in TFA containing 5% H_2O, 5%
anisole, 5% phenol and 5% EDT for 2 hours, giving a
crude product yield of 150 mg. Purification by
preparative HPLC of a 30 mg aliquot of crude material
was carried out using a gradient of 90 to 100% B over 40
minutes (A = 0.1 % TFA/water and B = MeOH) at a flow
rate of 9 mlmin. After lyophilisation, 4 mg of pure
material was obtained (analytical HPLC, gradient 90-100%
B over 20 minutes where B = MeOH, A = 0.01% TFA/water:
detection - UV 214 nm - product retention time = 23
minutes). Further product characterisation was carried
out using MALDI mass spectrometry: expected M+H at 2083,
found at 2088.

b)   Synthesis of a branched methotrexate core structure
containing a thiol moiety

5

10
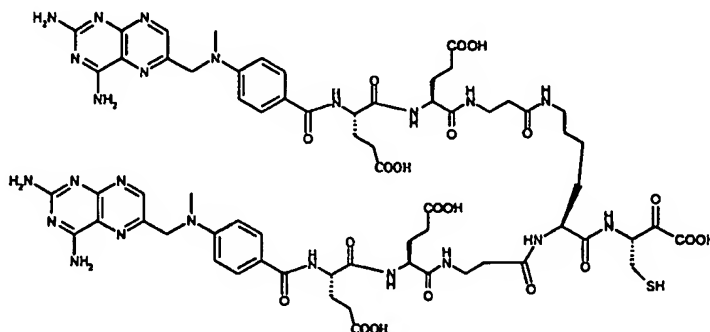
The methotrexate structure was synthesised on an ABI
433A automatic peptide synthesiser starting with Fmoc-
Cys(Trt) Tentagel resin on a 0.1 mmol scale.   The
15   simultaneous removal of product from the resin and
deprotection of protecting groups was carried out in TFA
containing 5% EDT and 5% H_2O for 2 hours, giving a crude
product yield of 160 mg.   Purification by preparative
HPLC of a 30 mg aliquot of crude material was carried
20   out using a gradient of 10 to 30% B over 40 minutes (A =
0.1% TFA/water and B = 0.1% TFA/acetonitrile) and a flow
rate of 9 ml/min.   After lyophilisation of the pure
fractions, 9 mg of pure material was obtained
(analytical HPLC, gradient 5-50% B where B = 0.1%
25   TFA/acetonitrile, A= 0.01% TFA/water: detection - UV 214
nm - product retention time = 9.5 minutes).   Further
product characterisation was carried out using MALDI
mass spectrometry: expected M+H at 1523, found at 1523.

30   c)   Preparation of multiple-specific gas-filled
microbubbles

DSPS (4.5 mg), thiol-containing lipopeptide from Example
64(a) (0.5 mg) and lipopeptide from (a) (0.2 mg) were
35   weighed into a clean vial and 1.0 ml of a solution of
1.4% propylene glycol/2.4% glycerol was added.   The
mixture was sonicated for 3-5 minsutes, warmed to 80°C

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for 5 minutes and then filtered through a 4.5 micron
filter. The mixture was cooled to room temperature and
the head space was flushed with perfluorobutane gas.
The vial was shaken in a cap mixer for 45 seconds and
the resulting microbubbles were centrifuged at 1000 rpm
for 3 minutes, whereafter the infranatant was discarded.

d) Conjugation of methotrexate branched structure to
thiolated microbubbles

The methotrexate structure from (b) above (0.5 mg) was
dissolved in PBS, pH 8.0. The solution was then added
to the thiol-containing microbubbles from (c) and
disulphide bond formation was allowed to proceed for 16
hours. Following extensive washing with PBS and water
the bubbles were analysed by microscopy and MALDI MS.

The disulphide bond linking the methotrexate structure
to the microbubbles may be reduced *in vivo* to liberate
the free drug molecule, so that such microbubbles in
combination with a tumour specific vector comprise a
drug delivery system. A physiologically acceptable
reducing agent such as glutathione may be used to bring
about drug release.

Example 34 - Preparation of gas-filled microbubbles
coated with poly-L-lysine complexed to fluorescein-
labeled DNA fragments from plasmid pBR322

This example is directed to the preparation of
microbubbles for gene therapy/anti-sense applications.
Specific targeting may be achieved by further doping of
microbubble membranes with vector-modified lipid
structures as described in Example 21.

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a) Preparation of DSPS-encapsulated gas-filled
microbubbles

DSPS (4.5 mg) was weighed into a clean vial. 1.0 ml of
a solution of 1.4% propylene glycol/2.4% glycerol was
added and the mixture was sonicated for 2 minutes and
then warmed to 80°C for 5 minutes. Immediately
following warming the solution was filtered through a 4
micron filter. The sample was cooled to room
temperature and the head space was flushed with
perfluorobutane gas. The vial was shaken in a cap mixer
for 45 seconds. The resulting microbubbles were then
washed once with deionised water and the infranatant was
discarded. The microbubbles were then resuspended in
0.5 ml water.

b) Preparation of poly-L-lysine/DNA complex and loading
of DSPS-encapsulated microbubbles

To 1 mg of poly-L-lysine (70-150 kD) in a clean vial was
added 0.1 ml of a fluorescein-labeled digest of plasmid
pBR322 dissolved in TE buffer (10 mM tris-HCl, pH 8).
The solution was made up to a total of 0.6 ml by
addition of water and the pH was adjusted to 8.
Complexing was allowed to proceed for 1 hour, after
which 0.05 mL of the polylysine-DNA solution was added
to the microbubble suspension from (a) above. After 1
hour microscopy was used to show that the bubbles were
fluorescent, confirming the presence of DNA.

Example 35 - Preparation of gas-filled microbubbles
containing a branched core peptide comprising a
Dabsylated-atherosclerotic plaque-binding sequence and
RGDS

This example is directed to the preparation of

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microbubbles having a thiol group on the surface for modification with thiol-containing vectors for targeting/drug delivery and drug release.

5   a)   Synthesis of the branched peptide Dabsyl-Tyr-Arg-
Ala-Leu-Val-Asp-Thr-leu-Lys-Lys(NH₂-Arg-Gly-Asp-Ser)-Gly-
Cys.OH

10

15
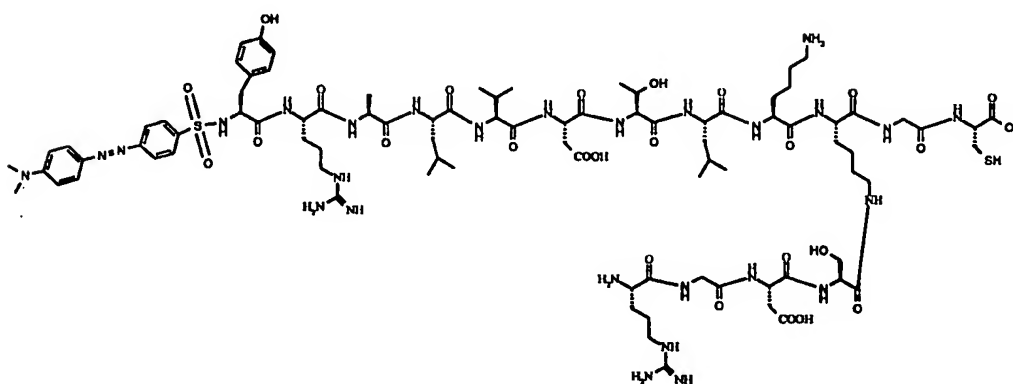
20   The peptide was synthesised on an ABI 433A automatic
peptide synthesiser starting with Fmoc-Cys(Trt)-Tentagel
resin on a 0.1 mmol scale using 1 mmol amino acid
cartridges. All amino acids were preactivated using
HBTU before coupling. The simultaneous removal of ·
25   peptide from the resin and side-chain protecting groups
was carried out in TFA containing 5% phenol, 5% EDT and
5% H₂O for 2 hours, giving a crude product yield of 160
mg. P urification by preparative HPLC of a 30 mg aliquot
of crude material was carried out using a gradient of 10
30   to 60% B over 40 minutes (where A = 0.1% TFA/water and B
= acetonitrile) at a flow rate of 9 ml/min. After
lyophilisation, 2.5 mg of pure material was obtained
(analytical HPLC, gradient 10-50% B over 20 minutes
where B = 0.1% TFA/acetonitrile and A = 0.01% TFA/water:
35   detection - UV 214 and 435 nm - product retention time =
21 minutes). Further product characterisation was
carried out using MALDI mass spectrometry: expected M+H

at 2070, found at 2073.

b) Preparation of thiol-containing gas-filled microbubbles

These were prepared as described in Example 64(a).

c) Oxidative coupling of thiolated microbubbles with multiple-specific peptide via disulphide bond formation

The infranatant from the microbubbles from (b) above was discarded and replaced with a solution of dabsyl-peptide from (a) (1 mg) in 0.7 ml dilute ammonia solution (pH 8). To this was added 0.2 ml of a stock solution containing 6 mg of potassium ferricyanate dissolved in 2 ml of water. The vial was placed on a roller table and thiol oxidation allowed to proceed for 2 hours. The bubbles were then washed extensively with water until the infranatant was free of the dabsyl-peptide as evidenced by HPLC and MALDI MS. Detection of microbubble-bound peptide was carried out by reduction of the disulphide bond using the water souble reducing agent tris-(2-carboxyethyl)-phosphine. Following reduction, the infranatant was found to contain free dabsyl-peptide as evidenced by HPLC and MALDI MS.

Other physiologically acceptable reducing agents such as reduced glutathione may also be useful for initiating release.

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Example 36 - Preparation of gas-filled microbubbles
encapsulated with DSPS and biotin-PEG$_{3400}$-acyl-
phosphatidylethanolamine and functionalised with
streptavidin, oligonucleotide biotin-
GAAAGGTAGTGGGGTCGTGTGCCGG and biotinylated fibrin-anti-
polymerant peptide (Biotin-GPRPPERHQS.NH$_2$)

a) Synthesis of biotin-PEG$_{3400}$-acyl-phosphatidyl
ethanolamine

A mixture of dipalmitoyl phosphatidyl ethanolamine,
(21.00 mg, 0.03 mmol), biotin-PEG-CO$_2$-NHS, (100 mg, 0.03
mmol) and triethylamine (42 μl, 0.30 mmol) in a solution
of chloroform/methanol (3:1) was stirred at room
temperature for 2 hours. After evaporation of the
solvents under reduced pressure, the residue was flash
chromatographed (methylene chloride/methanol/water,
40:8:1). The product was obtained as a yellow gum (112
mg, 94%), and structure was verified by NMR and
MALDI-MS.

b) Binding of fluorescein-conjugated streptavidin to
gas-filled microbubbles

Gas-filled microbubbles were prepared by mixing DSPS and
biotin-PEG$_{3400}$-acyl-phosphatidylethanolamine as described
in previous examples. The microbubble suspension was
divided into 0.2 ml aliquots and fluorescein-conjugated
streptavidin was added as shown in the table below. The
samples were incubated on a roller table for 15 or 30
minutes at ambient temperature before removal of excess
protein by washing in PBS. The samples were analysed by
flow cytometry and Coulter Counter. The results are
summarized in the table below.

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Results:

Aliquot no.	Added Streptavidin (mg/200:1 sample)	Incubation time (amb. temp.)	% Fluorescent particles	Particle median diameter (microns)
1	0		2.0	-
2	0		-	12 (foam)
3	0.2 (3x10^{-9}mmol)	30 min	7.8	3.9
4	2 (3x10^{-8}mmol)	30 min	26.2	4.2
5	10 (1.5x10^{-7}mmol)	15 min	30.5	na
6	20 (3x10^{-7}mmol)	30 min	97.9	5.2
7	40 (6x10^{-7}mmol)	15 min	96.7	5.1
8 DSPS control	20 (3x10^{-7}mmol)	15 min	0.6	3.7

c) Conjugation of streptavin-coated microbubbles with the oligonucleotide biotin-GAAAGGTAGTGGGGTCGTGTGCCGG and biotinylated fibrin-anti-polymerant peptide biotin-GPRPPERHQS

The particles from aliquot no. 6 above were centrifuged and the supernatant was replaced with 1 ml PBS buffer, pH 7.5, containing 0.2 mg of biotin-GAAAGGTAGTGGGGTCGTGTGCCGG and 0.2 mg of biotin-GPRPPERHQS (prepared as in Example 27(b) and (c)). After incubation for 24 hours the particles were washed extensively with PBS and water.

Other biotinylated vectors or therapeutic agents may be conjugated to streptavidin- or avidin-coated

microbubbles using this procedure.

<u>Example 37 - Preparation of gas-filled microbubbles
encapsulated with DSPS and functionalised with a
thrombi-targeting lipopeptide and the thrombolytic
enzyme tissue plasminogen activator</u>

This example is directed at the preparation of thrombus
targeted ultrasound contrast agents comprising a
therapeutic thromolytic agent.

<u>a) Synthesis of a lipopeptide with affinity for thrombi
(Diplamitoyl-Lys-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-
Glu-Tyr-Leu-Gln.NH$_2$)</u>

The lipopeptide was synthesised on an ABI 433 A
automatic peptide synthesiser starting with Rink amide
resin on a 0.1 mmol scale using 1 mmol amino acid
cartridges. All amino acids and palmitic acid were
preactivated using HBTU before coupling. The
simultaneous removal of peptide from the resin and side-
chain protecting groups was carried out in TFA
containing 5% phenol, 5% EDT, 5% anisole and 5% H$_2$O for 2
hours, giving a crude product yield of 80 mg.
Purification by preparative HPLC of a 20 mg aliquot of
the crude material was carried out. After
lyophilisation, 6 mg of pure material was obtained. The
product was characterised by MALDI mass spectrometry and
analytical HPLC.

b) Modification of tissue plasminogen activator with Sulpho-SMPB

A solution of 0.1 ml of ammonim carbonate buffer
5   containing 0.1 mg of t-PA was made up to 0.2 ml by the
addition of water. To this solution was added 0.4 mg of
Sulpho-SMPB (dissolved in 0.05 ml DMSO. The protein
solution was left standing at room temperature for 45
minutes, whereafter purification was carried out on a
10   Superdex 200 column. The product was eluted in PBS and
the modified protein fraction was collected.

c) Preparation of gas-filled microbubbles encapsulated with DSPS/thrombi-binding lipopeptide and thiol-
15   containing lipoeptide and conjugation of modified tissue plasminogen activator

DSPS (5.0 mg) was weighed into a clean vial along with
0.5 mg of the lipopeptide from (a) and 0.5 mg of the
20   thiol-containing lipopeptide from Example 64(a). To
this was added 1.0 ml of a solution of 1.4% propylene
glycol/2.4% glycerol and the mixture was sonicated for 2
minutes and then warmed to 80°C for 5 minutes.
Immediately following warming, the solution was filtered
25   through a 4 micron filter. The sample was cooled to
room temperature and the head space flushed with
perfluorobutane gas. The vial was shaken in a cap mixer
for 45 seconds and the resulting microbubbles were
washed twice with deionised water. The infranatant was
30   discarded and replaced with a 1 ml aliquot of the
protein solution from (b) above. The conjugation
reaction was allowed to proceed for 1 hour. The
microbubbles were centrifuged and the infranatant was
exchanged with a further 1 ml of protein solution. The
35   incubation step was repeated until all protein solution
was used up. The microbubbles were then washed
extensively with water and analysed by Coulter counter.

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The microbubbles were tested in the flow chamber assay
described in Example 21(c). Microbubbles modified with
protein were found to bind in higher numbers than those
comprising either lipopeptide/DSPS or DSPS alone.

The targeting/therapeutic/ultrasound activities of these
microbubbles be evaluated in models of both *in vitro* and
in vivo thrombogenisis.

Example 38 - Preparation of gas-filled microbubbles
comprising DSPS loaded with a lipopeptide comprising a
helical peptide with affinity for cell membranes

This example is directed to the preparation of targeted
microbubbles comprising a peptidic vector for targeting
of cell membrane structures.

a) Synthesis of a lipopeptide comprising a helical
peptide with affinity for cell membranes
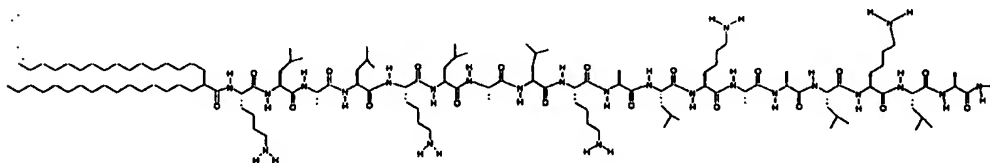
The lipopeptide was synthesised on an ABI 433A automatic
peptide synthesiser starting with Rink amide resin on a
0.2 mmol scale using 1 mmol amino acid cartridges. All
amino acids and 2-n-hexadecylstearic acid were
preactivated using HBTU before coupling. The
simultaneous removal of lipopeptide from the resin and
side-chain protecting groups was carried out in TFA
containing 5% H$_2$O for 2 hours, giving a crude product
yield of 520 mg. Purification by preparative HPLC of a
30 mg aliqout of crude material was carried out using a

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gradient of 90 to 100% B over 40 minutes (A = 0.1%
TFA/water and B = MeOH) at a flow rate of 9 ml/min.
After lyophilisation, 10 mg of pure material was
obtained (analytical HPLC, gradient 90-100% B over 20
5   minutes where B = MeOH, A= 0.01% TFA/water: detection -
UV 214 nm - product retention time = 23 minutes).
Further product characterisation was carried out using
MALDI mass spectrometry: expected M+H at 2369, found at
2375.

10

b)   Preparation of gas-filled microbubbles

DSPS (4.5 mg) and lipopeptide from (a)(0.5 mg) were
weighed into a clean vial and 1.0 ml of a solution of
15   1.4% propylene glycol/2.4% glycerol was added. The
mixture was sonicated for 3-5 minutes, warmed to 80°C
for 5 minutes and then filtered through a 4.5 mm filter.
The mixture was cooled to room temperature and the head
space was flushed with perfluorobutane gas. The vial was
20   shaken in a cap mixer for 45 seconds and the resulting
microbubbles were centrifuged at 1000 rpm for 3 minutes.
The microbubbles were then washed with water until no
lipopeptide could be detected by MALDI-MS. Coulter
counter, acoustic attenuation and pressure stability
25   studies were performed. To an aliquot of the washed
bubbles (ca. 0.2 ml) was added methanol (0.5 ml), and
the mixture was placed in a sonicator bath for 2
minutes. The resulting clear solution, on analysis by
MALDI-MS, was found to contain the lipopeptide.

30

c)   In vitro and in vivo tests

The microbubbles had similar characteristics in vitro
and in vivo as was found for the microbubbles made in
35   Example 21.

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Example 39 - Gas-filled microbubbles encapsulated with phosphatidylserine and a biotinylated lipopeptide

a) Synthesis of lipopeptide dipalmitoyl-lysinyl-tryptophanyl-lysinyl-lysinyl-lysinyl(biotinyl)-glycine

The lipopeptide was synthesised on an ABI 433A automatic peptide synthesiser starting with Fmoc-Gly-Wang resin on a 0.1 mmol scale using 1mmol amino acid cartridges. All amino acids and palmitic acid were preactivated using HBTU before coupling. The simultaneous removal of peptide from the resin and side-chain protecting groups was carried out in TFA containing 5% phenol, 5% EDT, 5% anisole and 5% H_2O for 2 hours, giving a crude product yield of 150 mg. Purification by preparative HPLC of a 40 mg aliqout of crude material was carred out using a gradient of 70 to 100% B over 40 minutes (A = 0.1% TFA/water and B = MeOH) at a flow rate of 9 ml/min. After lyophilisation. 14 mg of pure material (analytical HPLC, gradient 70-100% B where B = MeOH, A = 0.01% TFA/water: detection - UV 260 and fluorescence, Ex280, Em350 - product retention time = 22 minutes). Further product characterisation was carried out using MALDI mass spectrometry: expected M+H at 1478, found at 1471.

b) Preparation of gas-filled microbubbles comprising DSPS `doped' with the biotinylated lipopeptide sequence from (a)

DSPS (4.5 mg) and lipopeptide from (a) (0.5 mg, 0.2 mmol) were weighed into each of two vials, and 0.8 ml of a solution of 1.4% propylene glycol/2.4% glycerol was added to each vial. The mixtures were warmed to 80°C for 5 minutes (vials shaken during warming). The samples were cooled to room temperature and the head spaces were flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 seconds and then

rolled overnight. The resulting microbubbles were
washed several times with deionised water and analysed
by Coulter counter and acoustic attenuation. MALDI mass
spectral analysis was used to confirm incorporation of
5   lipopeptide into DSPS microbubbles as follows: ca. 50-
100 ml of microbubbles were transferred to a clean vial
and 50-100 ml water were added. The mixture was
sonicated for 30 seconds and spotted onto a clean target
disc (1 ml + 0.5 ml ACH matrix). Positive mode gave M+H
10   at 1474, expected for lipopeptide at 1478.

Example 40 - Preparation of multiple-specific gas-filled
microbubbles comprising DSPS loaded with a lipopeptide
15   comprising a non-bioactive interleukin-1 receptor-
binding peptide

This example is directed to the preparation of targeted
microbubbles comprising a non-bioactive peptidic vector
20   for targeting at the IL-1 recptor which does not induce
signal tranduction or prevent IL-1 binding.

a) Synthesis of a lipopeptide comprising a non-
bioactive interleukin-1 receptor-binding peptide
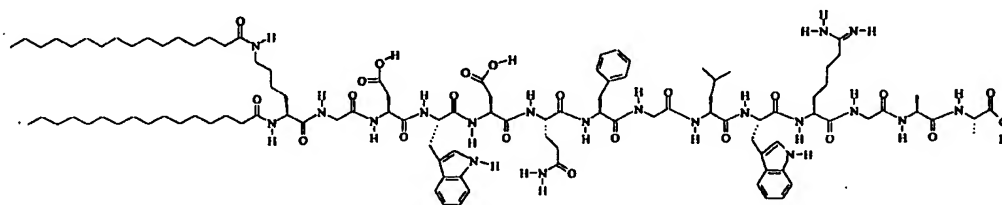
25

30

The lipopeptide was synthesised on an ABI 433A automatic
peptide synthesiser starting with Fmoc-Ala-Wang resin on
a 0.1 mmol scale using 1 mmol amino acid cartridges.
35   All amino acids and palmitic acid were preactivated
using HBTU before coupling. The simultaneous removal of
lipopeptide from the resin and side-chain protecting

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groups was carried out in TFA containing 5% H_2O, 5% anisole, 5% phenol and 5% EDT for 2 hours, giving a crude product yield of 150 mg. Purification by preparative HPLC of a 30 mg aliquot of crude material was carried out using a gradient of 90 to 100% B over 40 minutes (A = 0.1% TFA/water and B = MeOH) at a flow rate of 9 ml/min. After lyophilisation, 4 mg of pure material was obtained (analytical HPLC, gradient 90-100% B over 20 minutes where B = MeOH, A = 0.01% TFA/water: detection - UV 214 nm - product retention time = 23 minutes). Further product characterisation was carried out using MALDI mass spectrometry: expected M+H at 2083, found at 2088.

b) Preparation of gas-filled microbubbles

DSPS (4.5 mg) and lipopeptide from (a) (0.5 mg) were weighed into a clean vial and 1.0 ml of a solution of 1.4% propylene glycol/2.4% glycerol was added. The mixture was sonicated for 3-5 mins, warmed to 80°C for 5 minutes and then filtered through a 4.5 micron filter. The mixture was cooled to room temperature and the head space was flushed with perfluorobutane gas. The vials were shaken in a cap mixer for 45 seconds and the resulting microbubbles were centrifuged at 1000 rpm for 3 minutes. The microbubbles were then washed with water until no lipopeptide could be detected by MALDI-MS. To the washed microbubbles (ca. 0.2 ml) was added methanol (0.5 ml), and the mixture was placed in a sonicator bath for 2 minutes. The resulting clear solution, on analysis by MALDI-MS, was found to contain lipopeptide (expected 2083, found 2088).

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Example 41 - Preparation of perfluoropropane-filled
microbubbles comprising DSPC, DSPS and endothelial cell-
binding lipopeptide for targeted ultrasound imaging

To 0.8 ml of a solution containing DSPC:DSPS (3:1) (5mg/
ml) in propylene glycol/glycerol (4% in water) was added
0.5 mg of the lipopeptide from Example 31(b). The
mixture was heated to 80°C for 5 minutes and shaken. The
solution was then cooled to ambient temperature and the
headspace was flushed with perfluoropropane. The vial
was shaken on a cap-mixer for 45 seconds and placed on a
roller table for 5 minutes. The sample was centrifuged
at 2000 rpm for 5 minutes and the infranatant was
removed and replaced with distilled water. The
headspace was again flushed with perfluoropropane and
the sample was kept on a roller table until a
homogeneous appearance was obtained. The washing
procedure was repeated. The resulting ultrasound
contrast agent was characterised by Coulter counter
analysis, acoustic attenuation measurements and
resistance to external pressure. The microbubbles were
tested in the *in vitro* assay as detailed in Example 21.
A gradual accumulation of microbubbles binding to the
cells was observed.

Example 42 - Preparation of sulphur hexafluoride-
containing microbubbles comprising DSPC, DSPS and
endothelial cell-binding lipopeptide for targeted
ultrasound imaging

To 0.8 ml of a solution containing DSPC:DSPS (3:1) (5mg/
ml) in propylene glycol/glycerol (4% in water) was added
0.5 mg of the lipopeptide from Example 31(b). The
mixture was heated to 80°C for 5 minutes and shaken. The
solution was then cooled to ambient temperature and the
headspace was flushed with sulphur hexafluoride gas.

The vial was shaken on a cap-mixer for 45 seconds and
placed on a roller table for 5 minutes. The sample was
centrifuged at 2000 rpm for 5 minutes and the
infranatant was removed and replaced with distilled
5   water. The headspace was again flushed with sulphur
hexafluoride and the sample was kept on a roller table
until a homogenous appearance was obtained. The washing
procedure was repeated.
The resulting ultrasound contrast agent was confirmed by
10   Coulter counter, acoustic attenuation measurements and
resistance to external pressure.

Example 43 - Preparation of gas-filled microbubbles
comprising DSPG and endothelial cell-binding lipopeptide
15   for targeted ultrasound imaging

To 0.8 ml of a solution containing DSPG (5mg/ml) in
propylene glycol/glycerol (4% in water) was added 0.5 mg
of the lipopeptide from Example 31(b). The mixture was
20   heated to 80°C for 5 minutes and shaken. The solution
was then cooled to ambient temperature and the headspace
was flushed with perfluorobutane. The vial was shaken
on a cap-mixer for 45 seconds and placed on a roller
table for 5 minutes. The sample was centrifuged at 2000
25   rpm for 5 minutes and the infranatant was removed and
replaced with distilled water. The headspace was again
flushed with perfluorobutane and the sample was kept on
a roller table until a homogenous appearance was
obtained. The washing procedure was repeated. The
30   resulting ultrasound contrast agent was characterised by
Coulter counter analysis, acoustic attenuation
measurements and resistance to external pressure. The
microbubbles were tested in the *in vitro* assay as
detailed in Example 21: a gradual accumulation of
35   microbubbles binding to the cells was observed.

Example 44 - Preparation of perfluoropropane-filled
microbubbles comprising DSPG and endothelial cell
binding lipopeptide for targeted ultrasound imaging

5   To 0.8 ml of a solution containing DSPG (5mg/ml) in
     propylene glycol/glycerol (4% in water) was added 0.5 mg
     of the lipopeptide from Example 31(b). The mixture was
     heated to 80°C for 5 minutes and then shaken. The
     solution was then cooled to ambient temperature and the
10   headspace was flushed with perfluoropropane. The vial
     was shaken on a cap-mixer for 45 seconds and placed on a
     roller table for 5 minutes. The sample was centrifuged
     at 2000 rpm for 5 minutes and the infranatant was
     removed and replaced with distilled water. The
15   headspace was again flushed with perfluorobutane and the
     sample was kept on a roller table until a homogeneous
     appearance was obtained. The washing procedure was
     repeated. The resulting ultrasound contrast agent was
     characterised by Coulter counter analysis, acoustic
20   attenuation measurements and resistance to external
     pressure. The microbubbles were tested in the *in vitro*
     assay as detailed in Example 21: a gradual accumulation
     of microbubbles binding to the cells was observed.

25

Example 45 - Preparation of sulphur hexafluoride-
containing microbubbles comprising DSPG and endothelial
cell-binding lipopeptide for targeted ultrasound imaging

30   To 0.8 ml of a solution containing DSPG (5mg/ml) in
     propylene glycol/glycerol (4% in water) was added 0.5 mg
     of the lipopeptide from Example 31(b). The mixture was
     heated to 80°C for 5 minutes and shaken. The solution
     was then cooled to ambient temperature and the headspace
35   was flushed with sulphur hexafluoride gas. The vial was
     shaken on a cap-mixer for 45 seconds and placed on a
     roller table for 5 minutes. The sample was centrifuged

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at 2000 rpm for 5 minutes and the infranatant was
removed and replaced with distilled water. The
headspace was again flushed with sulphur hexafluoride
and the sample was kept on a roller table until a
homogeneous appearance was obtained. The washing
procedure was repeated. The resulting ultrasound
contrast agent was characterised by Coulter counter
analysis, acoustic attenuation measurements and
resistance to external pressure.

Example 46- Targeted gas-filled microbubbles comprising
DSPS coated non-covalently with polylysine

DSPS (5 mg) was weighed into a clean vial along with
poly-L-lysine (0.2 mg). To the vial was added 1.0 ml of
a solution of 1.4% propylene glycol/2.4% glycerol. The
mixture was warmed to 80°C for 5 minutes. The sample
was cooled to room temperature and the head space
flushed with perfluorobutane gas. The vial was shaken
in a cap mixer for 45 seconds and the resulting
microbubbles were centrifuged at 1000 rpm for 3 minutes.
Following extensive washing with water, PBS and water,
the final solution was examined for polylysine content
using MALDI MS. No polypeptide material was observed in
the final wash solution. Acetonitrile (0.5 ml) was then
added and the microbubbles were sonicated until all
bubbles had burst. Analysis of the resulting solution
for polylysine was again carried out using MALDI MS.
The results were as follows:

	MALDI expected	MALDI found
Poly-L-lysine	786, 914, 1042, 1170	790, 919, 1048, 1177

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Example 47 - Preparation of functionalised gas-filled
microbubbles for targeted ultrasound imaging

This example is directed to the preparation of
5   microbubbles having a reactive group on the surface for
non-specific targeting, principally utilising disulphide
exchange reactions to effect binding to a multiplicity
of cellular targets.

10   a)   Synthesis of a thiol-functionalised lipid molecule

The lipid structure shown above was synthesised on an
20   ABI 433A automatic peptide synthesiser starting with
Fmoc-Cys(Trt)-Wang resin on a 0.25 mmol scale using 1
mmol amino acid cartridges. All amino acids and
palmitic acid were preactivated using HBTU coupling
25   chemistry. The simultaneous removal of peptide from the
resin and deprotection of side-chain protecting groups
was carried out in TFA containing 5% EDT and 5% H_2O for 2
hours, giving a crude product yield of 250 mg.
Purification by preparative HPLC of a 40 mg aliquot of
30   crude material was carried out using a gradient of 90 to
100% B over 50 minutes (A = 0.1% TFA/water and B = MeOH)
at a flow rate of 9 ml/min. After lyophilisation, 24 mg
of pure material was obtained (analytical HPLC, gradient
70-100% B where B = 0.1% TFA/acetonitrile, A = 0.01%
35   TFA/water: detection - UV 214 nm - product retention
time = 23 minutes). Further product characterisation
was carried out using MALDI mass spectrometry: expected

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M+H at 1096, found at 1099.

b)   Preparation of gas-filled microbubbles comprising
DSPS `doped' with a thiol-containing lipid structure

DSPS (4.5 mg) and the lipid structure from (a) above
(0.5 mg, 0.4 mmol) were weighed into a clean vial and
0.8 ml of a solution containing 1.4% propylene glycol/
2.4% glycerol in water was added. The mixture was
warmed to 80°C for 5 minutes (vial shaken during
warming) and filtered while still hot through a 40 mm
filter. The sample was cooled to room temperature and
the head space was flushed with perfluorobutane gas.
The vial was shaken in a cap mixer for 45 seconds and
then placed on roller table overnight. The resulting
microbubbles were washed several times with deionised
water and analysed for thiol group incorporation using
Ellmans Reagent.

Example 48 - Preparation of gas-filled microbubbles
comprising DSPS doped with a thrombus-binding
lipopeptide

a)   Synthesis of a lipopeptide with affinity for thrombi
(Diplamitoyl-Lys-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-
Glu-Tyr-Leu-Gln.NH₂)

The lipopeptide was synthesised on an ABI 433 A
automatic peptide synthesiser starting with Rink amide

resin on a 0.1 mmol scale using 1 mmol amino acid
cartridges. All amino acids and palmitic acid were
preactivated using HBTU before coupling. The
simultaneous removal of peptide from the resin and side-
chain protecting groups was carried out in TFA
containing 5% phenol, 5% EDT, 5% anisole and 5% H_2O for 2
hours, giving a crude product yield of 80 mg.
Purification by preparative HPLC of a 20 mg aliquot of
the crude material was carried out. After
lyophilisation, 6 mg of pure material were obtained.
The product was characterised by MALDI mass spectrometry
and analytical HPLC.

b)   Preparation of thromi-targeting ultrasound
microbubbles

DSPS (4.5 mg) and lipopeptide from (a) (1.0 mg) were
weighed into a vial and 0.8 ml of a solution of 1.4%
propylene glycol/2.4% glycerol was added. The mixture
was warmed to 80°C for 5 minutes and then filtered
through a 4 micron filter. After cooling to room
temperature the head space was flushed with
perfluorobutane gas. The vial was shaken in a cap mixer
for 45 seconds and the resulting microbubbles were
washed extensively with deionised water. The
microbubbles were characterised by microscopy and
Coulter counter analysis. MALDI-MS was used to confirm
the presence of lipopeptide as described in previous
examples.

Example 49 - Preparation of transferrin-coated gas-
filled microbubbles for targeted ultrasound imaging

a) Synthesis of a thiol-functionalised lipid molecule

The lipid structure shown above was synthesised on an
ABI 433A automatic peptide synthesiser starting with
Fmoc-Cys(Trt)-Wang resin on a 0.25 mmol scale using 1
mmol amino acid cartridges. All amino acids and
palmitic acid were preactivated using HBTU before
coupling. The simultaneous removal of peptide from the
resin and deprotection of side-chain protecting groups
was carried out in TFA containing 5% EDT and 5% H_2O for 2
hours, giving a crude product yield of 250 mg.
Purification by preparative HPLC of a 40 mg aliquot of
crude material was carried out using a gradient of 90 to
100% B over 50 minutes (A = 0.1% TFA/water and B = MeOH)
at a flow rate of 9 ml/min. After lyophilisation, 24 mg
of pure material was obtained (analytical HPLC, gradient
70-100% B where B = 0.1% TFA/acetonitrile, A = 0.01%
TFA/water: detection - UV 214 nm - product retention
time = 23 minutes). Further product characterisation
was carried out using MALDI mass spectrometry: expected
M+H at 1096, found at 1099.

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b) Preparation of gas-filled microbubbles comprising
DSPS `doped' with a thiol-containing lipid structure

DSPS (4.5 mg) and lipid structure from (a) above (0.5
mg, 0.4 mmol) were weighed into a clean vial and 0.8 ml
of a solution of 1.4% propylene glycol/2.4% glycerol was
added. The mixture was warmed to 80°C for 5 minutes
(vial shaken during warming) and filtered while still
hot through a 40 mm filter. The sample was cooled to
room temperature and the head space was flushed with
perfluorobutane gas. The vial was shaken in a cap mixer
for 45 seconds and then placed on roller table
overnight. The resulting microbubbles were washed
several times with deionised water and analysed for
thiol group incorporation using Ellmans Reagent.

c) Modification of transferrin with fluorescein-NHS and
Sulpho-SMPB

To 4 mg of transferrin (Holo, human) in PBS (1 ml) was
added 0.5 ml DMSO solution containing 1 mg Sulpho-SMPB
and 0.5 mg fluorescein-NHS. The mixture was stirred for
45 minutes at room temperature and then passed through a
Sephadex 200 column using PBS as eluent. The protein
fraction was collected and stored at 4°C prior to use.

d) Microbubble conjugation with transferrin

To the thiol-containing microbubbles from (b) was added
1 ml of the modified transferrin protein solution from
(c). After adjusting the pH of the solution to 9 the
conjugation reaction was allowed to proceed for 2 hours
at room temperature. Following extensive washing with
deionised water the microbubbles were analysed by
Coulter counter (97% between 1 and 5 mm) and
fluorescence microscopy (highly fluorescent
microbubbles).

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Example 50 - Gas-filled microbubbles comprising DSPS
incorporating PE-PEG$_{2000}$-Mal conjugated to thiolated
trypsin fluorescein

5   a)   Synthesis of Boc-NH-PEG$_{2000}$-DSPE (t-butyl carbamate
poly(ethylene glycol)distearoylphosphatidyl-
ethanolamine)

DSPE (31 mg) was added to a solution of Boc-NH-PEG$_{2000}$-SC
10   (150 mg) in chloroform (2 ml), followed by triethylamine
(33 µl). The mixture was stirred at 41 °C for 10 minutes
until the starting material had dissolved. The solvent
was rotary evaporated and the residue was taken up in
acetonitrile (5 ml). The resulting dispersion was
15   cooled to 4 °C and centrifuged, whereafter the solution
was filtered and evaporated to dryness. The structure
of the resulting product was confirmed by NMR.

b)   Synthesis of H$_2$N-PEG$_{2000}$-DSPE (amino-poly(ethylene
20   glycol)-distearoylphosphatidylethanolamine)

Boc-NH-PEG$_{2000}$-DSPE (167 mg) was stirred in 4 M
hydrochloric acid in dioxane (5 ml) for 2.5 hours at
ambient temperature. The solvent was removed by rotary
25   evaporation and the residue was taken up in chloroform
(1.5 ml) and washed with water (2 x 1.5 ml). The
organic phase was evaporated *in vacuo*. TLC analysis
(chloroform/methanol/water 13:5:0.8) gave a single
ninhydrin positive spot with Rf = 0.6; confirmation of
30   the structure was obtained by NMR.

c)   Synthesis of Mal-PEG$_{2000}$-DSPE (3-maleimidopropionate
poly(ethylene glycol)distearoylphosphatidyl-
ethanolamine)

35

A solution of N-succinimidyl-3-maleimidopropionate (5.6
mg, 0.018 mmol) in tetrahydrofuran (0.2 ml) was added to

H_2N-PEG_{2000}-DSPE (65 mg, 0.012 mmol) dissolved in
tetrahydrofuran (1 ml) and 0.1 M sodium phosphate buffer
pH 7.5 (2 ml). The mixture was warmed to 30 °C and the
reaction was followed to completion by TLC, whereafter
the solvent was removed *in vacuo*. The title material
was purified on a flash silica column using 80:20
chloroform:methanol as eluent. The structure of the
pure product was confirmed by NMR and mass spectrometry.

d) Preparation of gas-filled microbubbles of DSPS 'doped' with PE-PEG_{2000}-Mal

DSPS (4.5 mg) and PE-PEG_{2000}-Mal from (c) above (0.5 mg)
were weighed into a clean vial and 1 ml of a solution of
1.4% propylene glycol/2.4% glycerol was added. The
mixture was warmed to 80°C for 5 minutes and then
filtered through a 4.5 mm filter. The sample was cooled
to room temperature and the head space was flushed with
perfluorobutane gas. The vial was shaken in a cap mixer
for 45 seconds and the resulting microbubbles were
washed three times with distilled water.

e) Preparation of fluorescein-labelled trypsin

To 5 mg of trypsin in PBS (1 ml) was added 0.2 ml DMSO
solution containing 1 mg of fluorescein-NHS. The mixture
was stirred for 45 minutes at room temperature. A
Sephadex 200 column was then charged with the modified
protein mixture and product was eluted at a flow rate of
1 ml/min using PBS. The protein fraction (5 ml) was
collected and stored at 4°C.

f) Preparation of thiolated, fluorescein-labelled trypsin

To the protein fraction from (e) was added 1 mg of
Traut's reagent and the mixture stirred at room

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temperature for a further 1 hour. 4 ml of the Traut's-
modified product was then charged on a Sephadex 200
column and the product was eluted with PBS. The protein
fraction containing maximum fluorescent intensity was

5   collected in a total volume of 6 ml.

g) Conjugation of microbubbles with thiolated,
fluorescein-labelled trypsin

10   Microbubbles from (d) were incubated on a roller table
in 1 ml of protein solution from (f) above. The
conjugation was allowed to proceed at pH 7.3-7.8 for 10
minutes before centrifugation and removal of the
infranatant. The process was repeated a further three

15   times, after which the bubbles were washed four times
with water to remove unconjugated protein.

D. Bubbles contained active enzyme as evidenced by the cleavage of
an Arg-pNA derivative in PBS.

20

E. Analysis of the bubbles by Coulter and measurement of
echogenicity was carried out.

Bubbles were pressure stable.

	FEK-022-015
Total concentration	0.83
Diameter 1-3 mm	40
Diameter 3-5 mm	28
Diameter 5-7mm	13
Freq of max Atten.	3.3
Atten at 2. Mhz	4.9
Atten at 3.5 Mhz	7.8
Atten at 5.0 MHz	7.2

Fig. 2 of the accompanying drawings represents flow
cytometry data showing a comparison with negative
control bubbles (left curve). 98% of the bubbles were
calculated to be fluorescent.

5

Example 51 - Gas-filled microbubbles comprising DSPS and
a captopril-containing molecule for diagnostic and
therapeutic applications

10

a) Synthesis of a lipopeptide functionalised with
captopril

15
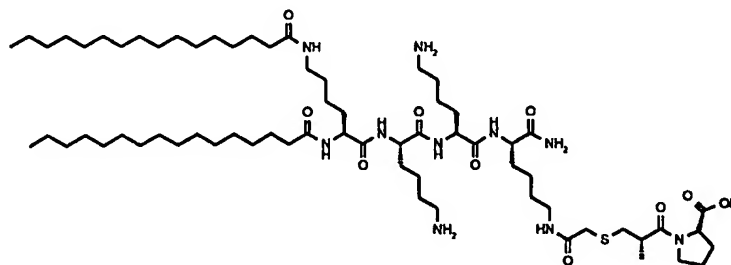
20 The structure shown above was synthesised by the manual
`bubbler' method starting with Fmoc-protected Rink Amide
MBHA resin on a 0.125 mmol scale. Coupling was carried
out using standard TBTU/HOBt/DIEA protocol. Bromoacetic

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acid was coupled through the side-chain of Lys as a
symmetrical anhydride using DIC preactivation.
Captopril dissolved in DMF was introduced on the solid
phase using DBU as base. Simultaneous removal of the
5   peptide from the resin and deprotection of side-chain
protecting groups was carried out in TFA containing 5%
EDT, 5% water and 5% ethyl methyl sulphide for 2 hours.
An aliquot of 10 mg of the crude material was purified
by preparative liquid chromatography using a gradient of
10   70 to 100% B over 60 minutes (A = 0.1% TFA/water and B =
0.1% TFA/acetonitrile) at a flow rate of 10 ml/min.
After lyophilisation, a yield of 2 mg of pure material
was obtained (analytical HPLC, gradient 70-100% B over
20 minutes, A = 0.1% TFA/water and B = 0.1%
15   TFA/acetonitrile, flow rate 1 ml/min, detection UV 214
nm, retention time 26 minutes). Further
characterisation was carried out using MALDI mass
spectrometry, giving M+H at 1265 as expected.

20   b) Preparation of gas-filled microbubbles comprising
DSPS and a compound containing captopril

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (4.5 mg) and product
25   from (a) (0.5 mg) in a vial. The mixture was sonicated
for 5 minutes and then warmed to 80 °C for 5 minutes
(vial was shaken during warming). The vial was then
cooled and the head space was flushed with
perfluorobutane gas. The vial was shaken in a cap mixer
30   for 45 seconds and the resulting microbubbles were
extensively washed with deionised water. MALDI mass
spectrometry showed no detectable level of compound from
(a) in the final wash solution. Incorporation of
captopril-containing lipopeptide into the microbubbles
35   was confirmed by MALDI-MS as follows: ca. 50 μl of
microbubbles were transferred to a clean vial containing
ca. 100 μl of 90% methanol. The mixture was sonicated

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for 30 seconds and analysed by MALDI mass spectrometry,
giving a M+H peak corresponding to lipopeptide from (a).

5   Example 52 - Gas-filled microbubbles comprising DSPS and
a vector with affinity for adrenergic receptors for
diagnostic and therapeutic applications

a) Synthesis of a protected atenolol derivative
10   suitable for solid phase coupling

i) Synthesis of methyl 4-[(2,3-epoxy)propoxy]-
phenylacetate

15   A mixture of methyl 4-hydroxyphenylacetate (4.98 g,
0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and
pyridine (121 µl, 1.5 mmol) was stirred at 85 °C for 2
hours. The reaction mixture was cooled and excess
epichlorohydrin was distilled off (rotavapor). The
20   residue was taken up in ethyl acetate, washed with brine
and dried (Na_2SO_4). The solution was filtered and
concentrated. The dark residue was chromatographed
(silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%)
of a colourless oil. ^1H (300 MHz) and ^{13}C NMR (75 MHz)
25   spectra were in accordance with the structure.

ii) Synthesis of methyl 4-[2-hydroxy-3-[(1-methylethyl)-
amino]propoxy]phenylacetate

30   A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate
(2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol)
and water (1.35 ml, 74.7 mmol) was stirred at room
temperature overnight. The reaction mixture was
concentrated (rotavapor) and the oily residue was
35   dissolved in chloroform and dried (Na_2SO_4). Filtration
and concentration gave quantitative yield of a yellow
oil that was used in the next step without further

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purification. The structure was verified by ^{1}H and ^{13}C NMR analysis.

iii) Synthesis of 4-[2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenylacetic acid hydrochloride

A solution of methyl 4-[2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenylacetate (563 mg, 2.00 mmol) in 6M hydrochloric acid (15 ml) was heated at 100 °C for 4hours. The reaction mixture was concentrated (rotavapor) and the residue was taken up in water and lyophilised. ^{1}H and ^{13}C NMR spectra were in accordance with the strucure and MALDI mass spectrometry gave a M+H at 268 as expected.

iv) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenylacetic acid

A solution of the 4-[2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenylacetic acid hydrochloride (2.0 mmol) in water (2 ml) was added to a solution of sodium bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15 ml). A solution of di-tert-butyl dicarbonate (0.48 g, 2.2 mmol) in dioxane (5 ml) was added. Progress of the reaction was monitored by TLC analysis (silica, CHCl$_3$/MeOH/AcOH 85:10:5), and portions of di-tert-butyl dicarbonate were added until conversion was complete. The reaction mixture was poured onto water saturated with potassium hydrogen sulphate and organic material was extracted into ethyl acetate. The organic phase was washed with water and brine, dried (Na$_2$SO$_4$) and filtered to give 0.6 g of crude material. The product was purified by chromatography (silica, CHCl$_3$/MeOH/AcOH 85:10:5). The solution was concentrated and the residue was taken up in glacial acetic acid and lyophilised. Yield 415 mg (56%), white solid. The structure was confirmed by ^{1}H and ^{13}C NMR analysis.

b) Synthesis of a lipopeptide functionalised with atenolol

The structure shown above was synthesised by the manual bubbler method starting with Fmoc-protected Rink Amide MBHA resin on a 0.125 mmol scale, using the compound from (a). Coupling was carried out using standard TBTU/HOBt/DIEA protocols. Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT and 5% water for 2 hours. Crude material was precipitated from ether and purified by preparative liquid chromatography using a gradient of 70 to 100% B over 60 minutes (A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate of 10 ml/min. After lyophilisation, a yield of 38 mg of pure material was obtained (analytical HPLC, gradient 70-100% B over 20 minutes, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1 ml/minute, detection UV 214 nm, retention time 25 minutes). Further characterisation was carried out using MALDI mass spectrometry (ACH matrix), giving M+H at 1258, expected 1257.

c) Preparation of gas-filled microbubbles comprising DSPS and a lipopeptide containig atenolol

A solution of 1.4% propylene glycol/2.4% glycerol (1.0 ml) was added to a mixture of DSPS (4.5 mg) and product

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from (b) (0.5 mg) in a vial. The mixture was sonicated
for 5 minutes, heated at 80 °C for 5 minutes (vial was
shaken during warming) and then cooled. The head space
was flushed with perfluorobutane gas and the vial was
shaken in a cap mixer for 45 seconds, whereafter the
contents were extensively washed with deionised water.
MALDI mass spectrometry showed no detectable level of
compound from (b) in the final wash solution.
Incorporation of atenolol-containing lipopeptide into
the microbubbles was confirmed by MALDI-MS as follows:
ca. 50 µl of microbubbles were transferred to a clean
vial containing ca. 100 µl of 90% methanol. The mixture
was sonicated for 30 seconds and analysed by MALDI-MS
(ACH-matrix), giving a M+H peak at 1259 corresponding to
lipopeptide (b).

d) In vitro analysis

The microbubbles were tested in the in vitro assay as
detailed in Example 21. A gradual accumulation of
microbubbles binding to the cells was observed.

Example 53 - Gas-filled microbubbles comprising DSPS and a lipopeptide consisting of a heparin sulphate-binding peptide (KRKR) and a fibronectin peptide (WQPPRARI) for targeting and a lipopeptide containing atenolol for therapeutic application

a) Synthesis of a lipopeptide consisting of a heparin sulphate-binding peptide (KRKR) and a fibronectin peptide (WQPPRARI)

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The lipopeptide was synthesised on an ABI 433A automatic
peptide synthesiser starting with Fmoc-Ile-Wang resin on
a 0.1 mmol scale using 1 mmol amino acid cartridges.
All amino acids and palmitic acid were preactivated
using HBTU before coupling. The simultaneous removal of
peptide from the resin and side-chain protecting groups
was carried out in TFA containing 5% phenol, 5% EDT, 5%
anisole and 5% H$_2$O for 2 hours, giving a crude product
yield of 150 mg. Purification by preparative HPLC of a
40 mg aliquot of crude material was carried out using a
gradient of 70 to 100% B over 40 minutes (A = 0.1%
TFA/water and B = MeOH) at a flow rate of 9 ml/min.
After lyophilisation, 16 mg of pure material was
obtained (analytical HPLC, gradient 70-100% B where B =
MeOH, A = 0.01% TFA/water: detection - UV 260 and
fluorescence, Ex280, Em350 - product retention time =
19.44 minutes). Further product characterisation was
carried out using MALDI mass spectrometry: expected M+H
at 2198, found at 2199.

b) Synthesis of a protected atenolol derivative
suitable for solid phase coupling

i) Synthesis of methyl 4-[(2,3-
epoxy)propoxy]phenylacetate

A mixture of methyl 4-hydroxyphenylacetate (4.98 g,
0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and
pyridine (121 μl, 1.5 mmol) was stirred at 85 °C for 2
hours. The reaction mixture was cooled, and excess

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epichlorohydrin was distilled off (rotavapor). The
residue was taken up in ethyl acetate, washed with brine
and dried (Na_2SO_4). The solution was filtered and
concentrated. The dark residue was chromatographed
5   (silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%)
of a colourless oil. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz)
spectra were in accordance with the structure.

ii) Synthesis of methyl 4-[2-hydroxy-3-[(1-methylethyl)-
10   amino]propoxy]phenylacetate

A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate
(2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol)
and water (1.35 ml, 74.7 mmol) was stirred at room
15   temperature overnight. The reaction mixture was
concentrated (rotavapor) and the oily residue was
dissolved in chloroform and dried (Na_2SO_4). Filtration
and concentration gave quantitative yield of a yellow
oil that was used in the next step without further
20   purification. The structure was verified by ^{1}H and ^{13}C
NMR analysis.

iii) Synthesis of 4-[2-hydroxy-3-[(1-methylethyl)-
amino]propoxy]phenylacetic acid hydrochloride
25

A solution of methyl 4-[2-hydroxy-3-[(1-methylethyl)-
amino]propoxy]phenylacetate (563 mg, 2.00 mmol) in 6M
hydrochloric acid (15 ml) was heated at 100 °C for 4
hours. The reaction mixture was concentrated
30   (rotavapor) and the residue was taken up in water and
lyophilised. ^{1}H and ^{13}C NMR spectra were in accordance
with the strucure and MALDI mass spectrometry gave a M+H
at 268 as expected.

35   iv) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methylethyl)-
amino]propoxy]phenylacetic acid

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A solution of the 4-[2-hydroxy-3-[(1-methylethyl)-
amino]propoxy]phenylacetic acid hydrochloride (2.0 mmol)
in water (2 ml) was added to a solution of sodium
bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15
ml). A solution of di-tert-butyl dicarbonate (0.48 g,
2.2 mmol) in dioxane (5 ml) was added. Progress of the
reaction was monitored by TLC analysis (silica,
CHCl₃/MeOH/AcOH 85:10:5), and portions of di-tert-butyl
dicarbonate were added until conversion was complete.
The reaction mixture was poured onto water saturated
with potassium hydrogen sulphate and organic material
was extracted into ethyl acetate. The organic phase was
washed with water and brine, dried (Na₂SO₄) and filtered
to give 0.6 g of crude material. The product was
purified by chromatography (silica, CHCl₃/MeOH/AcOH
85:10:5). The solution was concentrated and the residue
was taken up in glacial acetic acid and lyophilised.
Yield 415 mg (56%), white solid. The structure was
confirmed by ¹H and ¹³C NMR analysis.

c) Synthesis of a lipopeptide functionalised with
atenolol

The structure shown above was synthesised by the manual
bubbler method starting with Fmoc-protected Rink Amide
MBHA resin on a 0.125 mmol scale, using appropriate
amino acids, palmitic acid and the compound from (a).
Coupling was carried out using standard TBTU/HOBt/DIEA
protocols. Simultaneous removal of the peptide from the
resin and deprotection of side-chain protecting groups
was carried out in TFA containing 5% EDT and 5% water

for 2 hours. Crude material was precipitated from ether
and purified by preparative liquid chromatography using
a gradient of 70 to 100% B over 60 minutes (A = 0.1%
TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate
of 10 ml/min. After lyophilisation, a yield of 38 mg of
pure material was obtained (analytical HPLC, gradient
70-100% B over 20 minutes, A = 0.1% TFA/water and B =
0.1% TFA/acetonitrile, flow rate 1 ml/minute, detection
UV 214 nm, retention time 25 minutes). Further
characterisation was carried out using MALDI mass
spectrometry (ACH matrix), giving M+H at 1258, expected
1257.

d) Preparation of gas-filled microbubbles comprising
DSPS and a lipopeptide consisting of a heparin sulphate-
binding peptide (KRKR), a fibronectin peptide (WQPPRARI)
and a lipopeptide containing atenolol

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (5.0 mg), product
from (a) (0.5 mg) and product from (c) (0.5 mg) in a
vial. The mixture was sonicated for 5 minutes and then
heated at 80 °C for 5 minutes (vial was shaken during
warming). The solution was filtered and cooled. The
head space was flushed with perfluorobutane gas and the
vial was shaken in a cap mixer for 45 seconds,
whereafter the contents were extensively washed with
deionised water. Incorporation of atenolol-containing
lipopeptide into the microbubbles was confirmed by
MALDI-MS as follows: ca. 50 μl of microbubbles were
transferred to a clean vial containing ca. 100 μl of 90%
methanol. The mixture was sonicated for 30 seconds and
analysed by MALDI-MS (ACH matrix), giving two M+H peaks
at 2202 and 1259, corresponding to lipopeptide (a) and
to lipopeptide (c) respectively.

e) _In vitro analysis_

The microbubbles were tested in the _in vitro_ assay as
detailed in eexample 21. A gradual accumulation of
microbubbles binding to the cells was observed.

Example 54 - Gas-filled microbubbles comprising DSPS and
a lipophilic derivative of atenolol with affinity for
adrenergic receptors for diagnostic and therapeutic
applications

a) Synthesis of N-hexadecyl-4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetamide

i) Synthesis of methyl 4-[(2,3-epoxy)propoxy]-
phenylacetate

A mixture of methyl 4-hydroxyphenylacetate (4.98 g,
0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and
pyridine (121 μl, 1.5 mmol) was stirred at 85 °C for 2
hours. The reaction mixture was cooled and excess
epichlorohydrin was distilled off (rotavapor). The
residue was taken up in ethyl acetate, washed with brine
and dried (Na_2SO_4). The solution was filtered and
concentrated. The dark residue was chromatographed
(silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%)
of a colourless oil. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz)
spectra were in accordance with the structure.

ii) Synthesis of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate

A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate
(2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol)
and water (1.35 ml, 74.7 mmol) was stirred at room
temperature overnight. The reaction mixture was

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concentrated (rotavapor) and the oily residue was
dissolved in chloroform and dried (Na$_2$SO$_4$). Filtration
and concentration gave quantitative yield of a yellow
oil that was used in the next step without further
purification. The structure was verified by ^{1}H and ^{13}C
NMR analysis.

iii) Synthesis of 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid hydrochloride

A solution of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate (563 mg, 2.00 mmol) in
6M hydrochloric acid (15 ml) was heated at 100 °C for 4
hours. The reaction mixture was concentrated
(rotavapor) and the residue was taken up in water and
lyophilised. ^{1}H and ^{13}C NMR spectra were in accordance
with the strucure and MALDI mass spectrometry gave a M+H
at 268 as expected.

iv) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid

A solution of the 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid hydrochloride (2.0
mmol) in water (2 ml) was added to a solution of sodium
bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15
ml). A solution of di-tert-butyl dicarbonate (0.48 g,
2.2 mmol) in dioxane (5 ml) was added. Progress of the
reaction was monitored by TLC analysis (silica,
CHCl$_3$/MeOH/AcOH 85:10:5), and portions of di-tert-butyl
dicarbonate were added until conversion was complete.
The reaction mixture was poured onto water saturated
with potassium hydrogen sulphate and organic material
was extracted into ethyl acetate. The organic phase was
washed with water and brine, dried (Na$_2$SO$_4$) and filtered
to give 0.6 g of crude material. The product was
purified by chromatography (silica, CHCl$_3$/MeOH/AcOH

85:10:5). The solution was concentrated and the residue
was taken up in glacial acetic acid and lyophilised.
Yield 415 mg (56%), white solid. The structure was
confirmed by ^{1}H and ^{13}C NMR analysis.

v) Synthesis of N'-Boc, N-hexadecyl-4-[2-hydroxy-3-
[(1-methylethyl)amino]propoxy]phenylacetamide

A solution of N-Boc-4-[2-hydroxy-3-[(1-methylethyl)-
amino]propoxy]phenylacetic acid (92 mg, 0.25 mmol) and
hexadecylamine (60 mg, 0.25 mmol) in DMF (5 ml) was
cooled to 0 °C. HOBt (39 mg, 0.25 mmol) and N-(3-
dimethylaminopropyl)-N'-ethylcarbodiimide
hydrochloride (water soluble carbodiimide) (48 mg, 0.25
mmol) were added. The reaction mixture was stirred at
0°C for 1 hour and then at room temperature overnight.
The reaction mixture was poured onto water (25 ml)
containing sodium carbonate (2.5 g) and sodium chloride
(4.0 g). Precipitated material was filtered off,
washed with water and taken up in chloroform. The
chloroform phase was washed with 5% sodium carbonate and
water and dried (Na$_2$SO$_4$). The solution was filtered and
concentrated to give 150 mg of yellow-white crude
material. The product was purified by column
chromatography (silica, chloroform/methanol 95:5) to
give 118 mg (80%) of white material. The structure was
verified by ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR. The
product was further characterised by MALDI mass
spectrometry, giving a M+Na peak at 614 as expected.

vi) Synthesis of N-hexadecyl-4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetamide

To a solution of N'-Boc-N-hexadecyl-4-[2-hydroxy-3-[(1-
methyl-ethyl)amino]propoxy]phenylacetamide (10 mg) in
dichloromethane (9 ml) was added trifluoroacetic acid (1
ml). The reaction mixture was stirred for 2 hours at

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room temperature. TLC (silica, chloroform/methanol
95:5) showed complete conversion of starting material.
Solvents were evaporated off and the residue was taken
up in water/acetonitrile and lyophilised to give a
quantitative yield of white solid material. The
structure was verified by ^{1}H (500 MHz) and ^{13}C (125 MHz)
NMR analysis and further characterised by MALDI mass
spectrometry, giving M+H at 492 and M+Na at 514 as
expected.

b) Preparation of gas-filled microbubbles comprising
DSPS and N-hexadecyl-4-[2-hydroxy-3-[(1-methylethyl)-
amino]propoxy]phenylacetamide for diagnostic and
therapeutic applications

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (4.5 mg) and N-
hexadecyl-4-[2-hydroxy-3-[(1-methylethyl)-
amino]propoxy]phenylacetamide (0.5 mg) in a vial. The
mixture was sonicated for 5 minutes and then heated at
80 °C for 5 minutes (vial was shaken during warming).
The solution was filtered and cooled. The head space
was flushed with perfluorobutane gas and the vial was
shaken in a cap mixer for 45 seconds, whereafter the
contents were extensively washed with deionised water.
Incorporation of compound from (a) into the microbubbles
was confirmed by MALDI-MS as follows; ca. 50 μl of
microbubbles were transferred to a clean vial containing
ca. 100 μl of 90% methanol. The mixture was sonicated
for 30 seconds and analysed by MALDI-MS, giving a M+H
peak at 492 corresponding to N-hexadecyl-4-[2-hydroxy-3-
[(1-methylethyl)amino]propoxy]phenylacetamide.

Example 55 - Gas-filled microbubbles encapsulated with
DSPS and a compound containing folic acid for diagnostic
applications
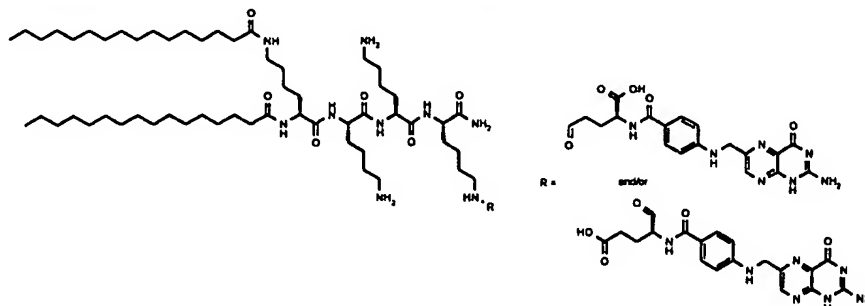
a)   Synthesis of a lipopeptide containing folic acid

The structure shown above was synthesised by the manual
bubbler method starting with Fmoc-protected Rink Amide
MBHA resin on a 0.125 mmol scale, using appropriate
amino acids, palmitic acid and folic acid.   Coupling was
carried out using standard TBTU/HOBt/DIEA protocols.
Simultaneous removal of the peptide from the resin and
deprotection of side-chain protecting groups was carried
out in TFA containing 5% EDT and 5% water for 2 hours.
Crude material was precipitated from ether and analysed
by MALDI mass spectrometry, giving a M+H peak
corresponding to the structure at 1435, expected 1430.
The material was further characterised by analytical
HPLC, gradient 70-100% B over 20 minutes, A = 0.1%
TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1.0
ml/minute, giving a product peak with retention time 27
minutes detected at UV 368 nm.

b) Preparation of gas-filled microbubbles comprising
DSPS and a lipopeptide containing folic acid

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (4.5 mg) and product
from (a) (0.5 mg) in a vial.   Dilute ammonia (to pH 8)
and DMSO (40 μl) were added and the mixture was
sonicated for 5 minutes and then heated at 80 °C for 5
minutes (vial was shaken during warming).   The solution
was filtered and cooled.   The head space was flushed

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with perfluorobutane gas and the vial was shaken in a
cap mixer for 45 seconds, whereafter the contents were
extensively washed with deionised water. Incorporation
of structure from (a) into the bubbles was confirmed by
5   MALDI-MS as follows: ca. 50 µl of microbubbles were
transferred to a clean vial containing ca. 100 µl of 90%
methanol. The mixture was sonicated for 30 seconds and
analysed by MALDI-MS (ACH matrix), giving a M+H peak at
1238 corresponding to structure from (a).

10

c)   *In vitro analysis*

The microbubbles were tested in the *in vitro* assay as
detailed in Example 21. A gradual accumulation of
15   microbubbles binding to the cells was observed.

Example 56 - Gas-filled microbubbles comprising DSPS and
a cholesteryl ester of chlorambucil for diagnostic and
20   therapeutic applications

a) Synthesis of cholesteryl 4-[4-[bis(2-
chloroethyl)amino]phenyl]butanoate

25   DIC (170 µl, 1.10 mmol) was added to a solution of
chlorambucil (669 mg, 2.20 mmol) in dry dichloromethane
(15 ml). The mixture was stirred at room temperature
for 0.5 hour and added to a solution of cholesterol (387
mg, 1.00 mmol) and DMAP (122 mg, 1.00 mmol) in
30   dichloromethane (10 ml). The reaction mixture was
stirred overnight and then poured onto 5% sodium
bicarbonate. The phases were separated and the organic
phase was washed with brine and dried (MgSO$_4$). The
solution was filtered and concentrated and the product
35   was purified by column chromatography (silica,
chloroform) to give 560 mg (83%)of colouless oil. The
product was characterised by MALDI mass spectrometry,

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giving M+H at 674 as expected. Further characterisation
was carried out using ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR
analysis, giving spectra in accordance with the
structure.

**b) Preparation of gas-filled microbubbles comprising
DSPS and a cholesteryl ester of chlorambucil for
diagnostic and/or therapeutic applications**

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (4.5 mg) and product
from (a) (0.5 mg) in a vial. The mixture was sonicated
for 5 minutes and then heated at 80 °C for 5 minutes
(vial was shaken during warming) and cooled. The head
space was flushed with perfluorobutane gas and the vial
was shaken in a cap mixer for 45 seconds, whereafter the
contents were extensively washed with deionised water.
MALDI mass spectrometry showed no detectable level of
compound from (a) in the final wash solution.
Incorporation of chlorambucil cholesteryl ester into the
bubbles was confirmed by MALDI-MS as follows: ca. 50 μl
of microbubbles were transferred to a clean vial
containing ca. 100 μl of 90% methanol. The mixture was
sonicated for 30 seconds and analysed by MALDI-MS,
giving a M+H peak at 668 corresponding to structure from
(a).

**Example 57 - Gas-filled microbubbles comprising DSPS and
a lipopeptide containing atenolol and a cholesterol
derivative of chlorambucil for diagnostic and
therapeutic applications**

**a) Synthesis of a protected atenolol derivative
suitable for solid phase coupling**

i) Synthesis of methyl 4-[(2,3-epoxy)propoxy]-

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phenylacetate

A mixture of methyl 4-hydroxyphenylacetate (4.98 g,
0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and
5   pyridine (121 μl, 1.5 mmol) was stirred at 85 °C for 2
hours. The reaction mixture was cooled and excess
epichlorohydrin was distilled off (rotavapor). The
residue was taken up in ethyl acetate, washed with brine
and dried (Na₂SO₄). The solution was filtered and
10   concentrated. The dark residue was chromatographed
(silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%)
of a colourless oil. ¹H (300 MHz) and ¹³C NMR (75 MHz)
spectra were in accordance with the structure.

15   ii) Synthesis of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate

A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate
(2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol)
20   and water (1.35 ml, 74.7 mmol) was stirred at room
temperature overnight. The reaction mixture was
concentrated (rotavapor) and the oily residue was
dissolved in chloroform and dried (Na₂SO₄). Filtration
and concentration gave quantitative yield of a yellow
25   oil that was used in the next step without further
purification. The structure was verified by ¹H and ¹³C
NMR analysis.

iii) Synthesis of 4-[2-hydroxy-3-[(1-methyl-
30   ethyl)amino]propoxy]phenylacetic acid hydrochloride

A solution of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate (563 mg, 2.00 mmol) in
6M hydrochloric acid (15 ml) was heated at 100 °C for 4
35   hours. The reaction mixture was concentrated
(rotavapor) and the residue was taken up in water and
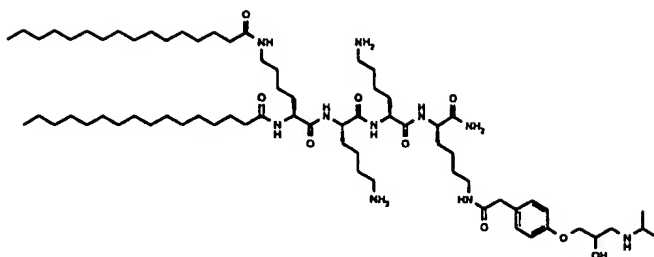lyophilised. ¹H and ¹³C NMR spectra were in accordance

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with the strucure and MALDI mass spectrometry gave a M+H
at 268 as expected.

iv) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid

A solution of the 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid hydrochloride (2.0
mmol) in water (2 ml) was added to a solution of sodium
bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15
ml). A solution of di-tert-butyl dicarbonate (0.48 g,
2.2 mmol) in dioxane (5 ml) was added. Progress of the
reaction was monitored by TLC analysis (silica,
CHCl₃/MeOH/AcOH 85:10:5), and portions of di-tert-butyl
dicarbonate were added until conversion was complete.
The reaction mixture was poured onto water saturated
with potassium hydrogen sulphate and organic material
was extracted into ethyl acetate. The organic phase was
washed with water and brine, dried (Na₂SO₄) and filtered
to give 0.6 g of crude material. The product was
purified by chromatography (silica, CHCl₃/MeOH/AcOH
85:10:5). The solution was concentrated and the residue
was taken up in glacial acetic acid and lyophilised.
Yield 415 mg (56%), white solid. The structure was
confirmed by ¹H and ¹³C NMR analysis.

b) Synthesis of a lipopeptide functionalised with
atenolol

The structure shown above was synthesised by the manual
bubbler method starting with Fmoc-protected Rink Amide
MBHA resin on a 0.125 mmol scale, using appropriate
amino acids, palmitic acid and the compound from (a).
Coupling was carried out using standard TBTU/HOBt/DIEA
protocols. Simultaneous removal of the peptide from the
resin and deprotection of side-chain protecting groups
was carried out in TFA containing 5% EDT and 5% water
for 2 hours. Crude material was precipitated from ether
and purified by preparative liquid chromatography using
a gradient of 70 to 100% B over 60 minutes (A = 0.1%
TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate
of 10 ml/min. After lyophilisation, a yield of 38 mg of
pure material was obtained (analytical HPLC, gradient
70-100% B over 20 minutes, A = 0.1% TFA/water and B =
0.1% TFA/acetonitrile, flow rate 1 ml/minute, detection
UV 214 nm, retention time 25 minutes). Further
characterisation was carried out using MALDI mass
spectrometry (ACH matrix), giving M+H at 1258, expected
1257.

c)   Synthesis of cholesteryl 4-[4-[bis(2-
chloroethyl)amino]phenyl]butanoate

DIC (170 μl, 1.10 mmol) was added to a solution of
chlorambucil (669 mg, 2.20 mmol) in dry dichloromethane
(15 ml). The mixture was stirred at room temperature for
0.5 hour and added to a solution of cholesterol (387 mg,
1.00 mmol) and DMAP (122 mg, 1.00 mmol) in
dichloromethane (10 ml). The reaction mixture was

stirred overnight and then poured onto 5% sodium
bicarbonate. The phases were separated and the organic
phase was washed with brine and dried (MgSO$_4$). The
solution was filtered and concentrated and the product

5   was purified by column chromatography (silica,
chloroform) to give 560 mg (83%) of colouless oil. The
product was characterised by MALDI mass spectrometry,
giving M+H at 674 as expected. Further characterisation
was carried out using ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR

10   analysis, giving spectra in accordance with the
structure.

d)   Preparation of gas-filled microbubbles comprising
DSPS and a lipopeptide containing atenolol and a

15   cholesteryl ester of chloambucil

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (5.0 mg), product
from (b) (0.5 mg) and product from (c) (0.5 mg) in a

20   vial. The mixture was sonicated for 5 minutes and then
warmed to 80 °C for 5 minutes (vial was shaken during
warming). The solution was filtered and cooled. The
head space was flushed with perfluorobutane gas and the
vial was shaken in a cap mixer for 45 seconds,

25   whereafter the contents were extensively washed with
deionised water. Incorporation of compounds (b) and (c)
into the microbubbles was confirmed by MALDI-MS as
follows: ca. 50 μl of microbubbles were transferred to a
clean vial containing ca. 100 μl of 90% methanol. The

30   mixture was sonicated for 30 seconds and analysed by
MALDI-MS (ACH-matrix), giving a M+H peak corresponding
to lipopeptide (b) and cholesteryl ester (c).

e)   In vitro analysis

35

The microbubbles were tested in the in vitro assay as
detailed in Example 21. A gradual accumulation of

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microbubbles binding to the cells was observed.

Example 58 - Gas-fiiled microbubbles comprising DSPS and
a lipopeptide containing atenolol for cell targeting and
a lipophilic thiol ester of captopril for therapeutic
use

a) Synthesis of a protected atenolol derivative suitable
for solid phase coupling

i) Synthesis of methyl 4-[(2,3-epoxy)propoxy]-
phenylacetate

A mixture of methyl 4-hydroxyphenylacetate (4.98 g,
0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and
pyridine (121 μl, 1.5 mmol) was stirred at 85 °C for 2
hours. The reaction mixture was cooled and excess
epichlorohydrin was distilled off (rotavapor). The
residue was taken up in ethyl acetate, washed with brine
and dried (Na$_2$SO$_4$). The solution was filtered and
concentrated. The dark residue was chromatographed
(silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%)
of a colourless oil. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz)
spectra were in accordance with the structure.

ii) Synthesis of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate

A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate
(2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol)
and water (1.35 ml, 74.7 mmol) was stirred at room
temperature overnight. The reaction mixture was
concentrated (rotavapor) and the oily residue was
dissolved in chloroform and dried (Na$_2$SO$_4$). Filtration
and concentration gave quantitative yield of a yellow
oil that was used in the next step without further

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purification.   The structure was verified by ^{1}H and ^{13}C
NMR analysis.

iii) Synthesis of 4-[2-hydroxy-3-[(1-methyl-
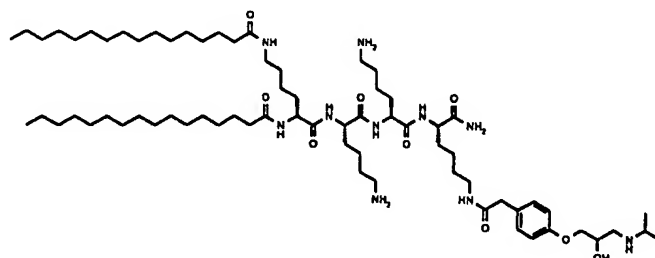ethyl)amino]propoxy]phenylacetic acid hydrochloride

A solution of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate (563 mg, 2.00 mmol) in
6M hydrochloric acid (15 ml) was heated at 100 °C for 4
hours.   The reaction mixture was concentrated
(rotavapor) and the residue was taken up in water and
lyophilised. ^{1}H and ^{13}C NMR spectra were in accordance
with the strucure and MALDI mass spectrometry gave a M+H
at 268 as expected.

iv) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid

A solution of the 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid hydrochloride (2.0
mmol) in water (2 ml) was added to a solution of sodium
bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15
ml).   A solution of di-tert-butyl dicarbonate (0.48 g,
2.2 mmol) in dioxane (5 ml) was added.   Progress of the
reaction was monitored by TLC analysis (silica,
CHCl$_3$/MeOH/AcOH 85:10:5), and portions of di-tert-butyl
dicarbonate were added until conversion was complete.
The reaction mixture was poured onto water saturated
with potassium hydrogen sulphate and organic material
was extracted into ethyl acetate.   The organic phase was
washed with water and brine, dried (Na$_2$SO$_4$) and filtered
to give 0.6 g of crude material.   The product was
purified by chromatography (silica, CHCl$_3$/MeOH/AcOH
85:10:5).   The solution was concentrated and the residue
was taken up in glacial acetic acid and lyophilised.
Yield 415 mg (56%), white solid.   The structure was
confirmed by ^{1}H and ^{13}C NMR analysis.

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b) Synthesis of a lipopeptide functionalised with atenolol

5

10

The structure shown above was synthesised by the manual bubbler method starting with Fmoc-protected Rink Amide MBHA resin on a 0.125 mmol scale, using appropriate amino acids, palmitic acid and the compound from (a).

15   Coupling was carried out using standard TBTU/HOBt/DIEA protocols. Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT and 5% water for 2 hours. Crude material was precipitated from ether

20   and purified by preparative liquid chromatography using a gradient of 70 to 100% B over 60 minutes (A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate of 10 ml/min. After lyophilisation, a yield of 38 mg of pure material was obtained (analytical HPLC, gradient

25   70-100% B over 20 minutes, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1 ml/minute, detection UV 214 nm, retention time 25 minutes). Further characterisation was carried out using MALDI mass spectrometry (ACH matrix), giving M+H at 1258, expected

30   1257.

c) Synthesis of cholanic acid thiol ester of captopril

35   A mixture of 5-β-cholanic acid (361 mg, 1.00 mmol) and DIC (77μl, 0.50 mmol) in dichloromethane (5 ml) was stirred for 10 minutes and then added to a solution of

captopril (130 mg, 0.600 mmol) and DBU (180 μl, 1.20
mmol) in dichloromethane (10 ml). The reaction mixture
was stirred overnight and then poured onto dilute
hydrochloric acid. Chloroform (30 ml) was added. The

5   phases were separated and the organic phase was washed
with water and brine and dried (MgSO₄). After filtration
and concentration, the crude material was
chromatographed (silica, chloroform/methanol/acetic acid
95:4:1). The product was lyophilised from a

10   acetonitrile/water/ethanol mixture. Yield 137 mg (49%)
of off-white solid. The structure was verified by ^{1}H
(500 MHz) and ^{13}C (125 MHz) NMR spectroscopy. Further
characterisation was carried out using MALDI mass
spectrometry, giving a M+Na peak in positive mode at m/z

15   584.

d) Preparation of gas-filled microbubbles comprising
DSPS and a lipopeptide containing atenolol for cell
targeting and a lipophilic thiol ester of captopril for

20   therapeutic use

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (5.0 mg) and products
from (b) (0.5 mg) and (c) (0.5 mg) in a vial. The

25   mixture was sonicated for 5 minutes and then heated at
80 °C for 5 minutes (vial was shaken during warming) and
cooled. Head space was flushed with perfluorobutane gas
and the vial was shaken in a cap mixer for 45 seconds
followed by extensive washing with deionised water.

30   MALDI mass spectrometry showed no detectable level of
compound from (b) or (c) in the final wash solution.
Incorporation of compounds from (b) and (c) into the
microbubbles was confirmed by MALDI-MS as follows: ca.
50 μl of microbubbles were transferred to a clean vial

35   containing ca. 100 μl of 90% methanol. The mixture was
sonicated for 30 seconds and analysed by MALDI-MS (ACH-
matrix), giving peaks according to structures from (b)

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and (c) respectively.

e) *In vitro analysis*

5   The microbubbles were tested in the *in vitro* assay as
detailed in Example 21. A gradual accumulation of
microbubbles binding to the cells was observed.

10   Example 59 - Gas-filled microbubbles comprising
phosphatidylserine and biotinamide-PEG-ß-Ala-cholesterol
and a cholesteryl ester of chlorambucil for diagnostic
and therapeutic applications

15   a) Synthesis of cholesteryl N-Boc-ß-alaninate

DIC (510 µl) was added to a solution of Boc-ß-Ala-OH
(1.25 g, 6.60 mmol) in dichloromethane (15 ml) under an
inert atmosphere. The reaction mixture was stirred for
20   30 minutes and then transferred to a flask containing a
solution of cholesterol (1.16 g, 3.00 mmol) and DMAP
(367 mg, 3.00 mmol) in dichloromethane (15 ml). The
reaction mixture was stirred for 2 hours and then poured
onto an aqeous solution of potassium hydrogen sulphate.
25   After phase separation the aqueous phase was extracted
with chloroform. The combined organic phases were
washed with aqueous potassium hydrogen sulphate and
water and dried (MgSO₄). After filtration and
evaporation the crude product was chromatographed
30   (silica, chloroform/methanol 99:1) to give 1.63 g (97%)
of white solid. The structure was confirmed by ^{1}H NMR
(500 MHz).

b) Synthesis of cholesteryl ß-alaninate hydrochloride
35

A solution of compound from (a) (279 mg, 0.500 mmol) in
1M hydrochloric acid in 1,4-dioxane (5 ml) was stirred

at room temperature for 4 hours. The reaction mixture
was concentrated to give a quantitative yield of
cholesteryl β-alaninate hydrochloride. The structure
was confirmed by ^{1}H NMR (500 MHz) analysis and by MALDI
5   mass spectrometry, giving a M+Na peak at 482, expected
481.

c)   Biotin-PEG$_{3400}$-β-Ala-Cholesterol

10   To a solution of cholesteryl β-alaninate hydrochloride
(15 mg, 0.03 mmol) in chloroform/wet methanol (2.6:1, 3
ml) was added triethylamine (42 μl, 0.30 mmol). The
mixture was stirred for 10 minutes at room temperature
and a solution of biotin-PEG$_{3400}$-NHS (100 mg, 0.03 mmol)
15   in 1,4-dioxane (1 ml) was added dropwise. After
stirring at room temperature for 3 hours the mixture was
evaporated to dryness and the residue was purified by
flash chromatography to give white crystals, yield 102
mg (89%). The structure was verified by MALDI-MS and by
20   NMR analysis.

d)   Synthesis of cholesteryl 4-[4-[bis(2-
chloroethyl)amino]phenyl]butanoate

25   DIC (170 μl, 1.10 mmol) was added to a solution of
chlorambucil (669 mg, 2.20 mmol) in dry dichloromethane
(15 ml). The mixture was stirred at room temperature
for 0.5 hour and added to a solution of cholesterol (387
mg, 1.00 mmol) and DMAP (122 mg, 1.00 mmol) in
30   dichloromethane (10 ml). The reaction mixture was
stirred overnight and then poured onto 5% sodium
bicarbonate. The phases were separated and the organic
phase was washed with brine and dried (MgSO$_4$). The
solution was filtered and concentrated and the product
35   was purified by column chromatography (silica,
chloroform) to give 560 mg (83%) yield of colouless oil.
The product was characterised by MALDI mass

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spectrometry, giving M+H at 674 as expected. Further
characterisation was carried out using ^{1}H (500 MHz) and
^{13}C (125 MHz) NMR analysis, giving spectra in accordance
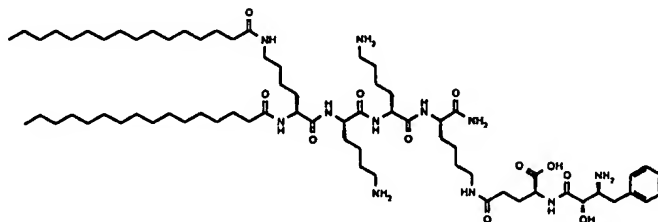with the structure.

e) Preparation of gas-filled microbubbles

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (5 mg) and products
from (c) (0.5 mg) and (d) (0.5 mg) in a vial. The
mixture was sonicated for 5 minutes and then heated at
80 °C for 5 minutes (vial was shaken during warming) and
cooled. The head space was flushed with perfluorobutane
gas and the vial was shaken in a cap mixer for 45
seconds, whereafter the contents were extensively washed
with deionised water. MALDI mass spectrometry showed no
detectable level of compound from (c) or (d) in the
final wash solution. Incorporation of compounds from
(c) and (d) into the microbubbles was confirmed by
MALDI-MS as follows: ca. 50 μl of microbubbles were
transferred to a clean vial containing ca. 100 μl of 90%
methanol. The mixture was sonicated for 30 seconds and
analysed by MALDI-MS (ACH-matrix), giving M+H peaks
corresponding to compounds from (c) and (d).

Example 60 - Gas-filled microbubbles comprising DSPS and
a lipopeptide containing a derivative of bestatin for
diagnostic and therapeutic applications

a) Synthesis of a lipopeptide containing a derivative of
bestatin

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The structure shown above was synthesised by the manual
bubbler method starting with Fmoc-protected Rink Amide
MBHA resin on a 0.125 mmol scale, using appropriate
amino acids and palmitic acid. Coupling was carried out
using standard TBTU/HOBt/DIEA protocols. Simultaneous
removal of the peptide from the resin and deprotection
of side-chain protecting groups was carried out in TFA
containing 5% EDT and 5% water for 2 hours. Crude
material was precipitated from ether and purified by
preparative liquid chromatography using a gradient of 70
to 100% B over 60 minutes (A = 0.1% TFA/water and B =
0.1% TFA/acetonitrile) at a flow rate of 10 ml/min.
After lyophilisation, a yield of 12 mg of pure material
was obtained (analytical HPLC, gradient 70-100% B over
20 minutes, A = 0.1% TFA/water and B = 0.1%
TFA/acetonitrile, flow rate 1 ml/minute, detection UV
214 nm, retention time 25 minutes). Further
characterisation was carried out using MALDI mass
spectrometry (ACH matrix), giving M+H at 1315, expected
1314.

b) Preparation of gas-filled microbubbles comprising
DSPS and a lipopeptide containing a derivative of
bestatin for diagnostic and therapeutic applications

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (4.5 mg) and product
from (a) (0.5 mg) in a vial. The mixture was sonicated
for 5 minutes and then heated at 80 °C for 5 minutes
(vial was shaken during warming) and cooled. The head
space was flushed with perfluorobutane gas and the vial
was shaken in a cap mixer for 45 seconds and the
contents were extensively washed with deionised water.
MALDI mass spectrometry showed no detectable level of
compound from (b) in the final wash solution.
Incorporation of atenolol-containing lipopeptide into
the microbubbles was confirmed by MALDI-MS as follows:

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ca. 50 µl of microbubbles were transferred to a clean
vial containing ca. 100 µl of 90% methanol. The mixture
was sonicated for 30 seconds and analysed by MALDI-MS
(ACH-matrix), giving a M+H peak at 1320, expected at
5   1314, corresponding to lipopeptide from (a).

c)   *In vitro* analysis

The microbubbles were tested in the *in vitro* assay as
10   detailed in Example 21. A gradual accumulation of
microbubbles binding to the cells was observed.

Example 61 - Gas-filled microbubbles comprising DSPS and
15   a lipopeptide containing chlorambucil for diagnostic and
therapeutic applications

a) Synthesis of a lipopeptide containing chlorambucil

20

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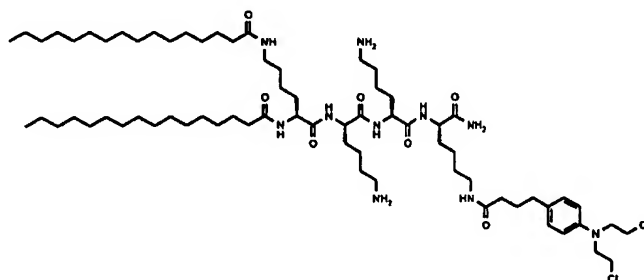
The structure shown above was synthesised by the manual
bubbler method starting with Fmoc-protected Rink Amide
MBHA resin on a 0.125 mmol scale, using appropriate
30   amino acids and palmitic acid. Coupling was carried out
using standard TBTU/HOBt/DIEA protocol. Chlorambucil
was coupled through the side-chain of Lys as a
symmetrical anhydride using DIC preactivation.
Simultaneous removal of the peptide from the resin and
35   deprotection of side-chain protecting groups was carried
out in TFA containing 5% EDT, 5% water and 5% ethyl
methyl sulphide for 2 hours. An aliqout of 10 mg of the

crude material was purified by preparative liquid
chromatography using a gradient of 70 to 100% B over 60
minutes (A = 0.1% TFA/water and B = 0.1%
TFA/acetonitrile) at a flow rate of 10 ml/min. After
lyophilisation, a yield of 30 mg of pure material was
obtained (analytical HPLC, gradient 70-100% B over 20
minutes, A = 0.1% TFA/water and B = 0.1%
TFA/acetonitrile, flow rate 1 ml/minute, detection UV
214 nm retention time 26.5 minutes). Further
characterisation was carried out using MALDI mass
spectrometry, giving M+H at 1295, expected 1294.

b)   Preparation of gas-filled microbubbles comprising
DSPS and a lipopeptide containing chlorambucil for
diagnostic and therapeutic applications

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (4.5 mg) and product
from (a) (0.5 mg) in a vial. The mixture was sonicated
for 5 minutes and then heated at 80 °C for 5 minutes
(vial was shaken during warming) and cooled. The head
space was flushed with perfluorobutane gas and the vial
was shaken in a cap mixer for 45 seconds, whereafter the
contents were extensively washed with deionised water.
MALDI mass spectrometry showed no detectable level of
compound from (a) in the final wash solution.
Incorporation of chlorambucil-containing lipopeptide
into the bubbles was confirmed by MALDI-MS as follows:
ca. 50 μl of microbubbles were transferred to a clean
vial containing ca. 100 μl of 90% methanol. The mixture
was sonicated for 30 seconds and analysed by MALDI-MS
(ACH-matrix), giving a M+H peak at 1300, expected at
1294 and a M+Na peak at 1324, expected 1317.

c)   *In vitro* analysis

The microbubbles were tested in the *in vitro* assay as

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detailed in Example 21. A gradual accumulation of
microbubbles binding to the cells was observed.

5   Example 62 - Gas-filled microbubbles comprising DSPS, a
lipopeptide containing atenolol and a lipophilic
derivative of captopril for diagnostic and therapeutic
applications

10   a)   Synthesis of a protected atenolol derivative
suitable for solid phase coupling

i) Synthesis of methyl 4-[(2,3-epoxy)propoxy]-
phenylacetate

15

A mixture of methyl 4-hydroxyphenylacetate (4.98 g,
0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and
pyridine (121 μl, 1.5 mmol) was stirred at 85 °C for 2
hours. The reaction mixture was cooled, and excess
20   epichlorohydrin was distilled off (rotavapor). The
residue was taken up in ethyl acetate, washed with brine
and dried (Na_2SO_4). The solution was filtered and
concentrated. The dark residue was chromatographed
(silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%)
25   of a colourless oil. ^1H (300 MHz) and ^{13}C NMR (75 MHz)
spectra were in accordance with the structure.

ii) Synthesis of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate

30

A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate
(2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol)
and water (1.35 ml, 74.7 mmol) was stirred at room
temperature overnight. The reaction mixture was
35   concentrated (rotavapor) and the oily residue was
dissolved in chloroform and dried (Na_2SO_4). Filtration
and concentration gave quantitative yield of a yellow

oil that was used in the next step without further
purification. The structure was verified by ^{1}H and ^{13}C
NMR analysis.

5   iii) Synthesis of 4-[2-hydroxy-3-[(1-methyl-
       ethyl)amino]propoxy]phenylacetic acid hydrochloride

       A solution of methyl 4-[2-hydroxy-3-[(1-methyl-
       ethyl)amino]propoxy]phenylacetate (563 mg, 2.00 mmol) in
10   6M hydrochloric acid (15 ml) was heated at 100 °C for 4
       hours. The reaction mixture was concentrated
       (rotavapor) and the residue was taken up in water and
       lyophilised. ^{1}H and ^{13}C NMR spectra were in accordance
       with the strucure and MALDI mass spectrometry gave a M+H
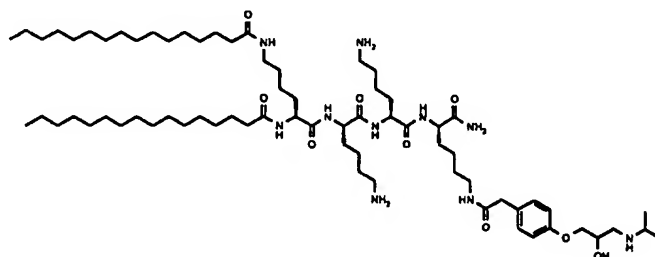15   at 268 as expected.

       iv) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methyl-
       ethyl)amino]propoxy]phenylacetic acid

20   A solution of the 4-[2-hydroxy-3-[(1-methyl-
       ethyl)amino]propoxy]phenylacetic acid hydrochloride (2.0
       mmol) in water (2 ml) was added to a solution of sodium
       bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15
       ml). A solution of di-tert-butyl dicarbonate (0.48 g,
25   2.2 mmol) in dioxane (5 ml) was added. Progress of the
       reaction was monitored by TLC analysis (silica,
       CHCl$_3$/MeOH/AcOH 85:10:5), and portions of di-tert-butyl
       dicarbonate were added until conversion was complete.
       The reaction mixture was poured onto water saturated
30   with potassium hydrogen sulphate and organic material
       was extracted into ethyl acetate. The organic phase was
       washed with water and brine, dried (Na$_2$SO$_4$) and filtered
       to give 0.6 g of crude material. The product was
       purified by chromatography (silica, CHCl$_3$/MeOH/AcOH
35   85:10:5). The solution was concentrated and the residue
       was taken up in glacial acetic acid and lyophilised.
       Yield 415 mg (56%), white solid. The structure was

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confirmed by ^{1}H and ^{13}C NMR analysis.

5

b) Synthesis of a lipopeptide functionalised with atenolol

10

The structure shown above was synthesised by the manual
bubbler method starting with Fmoc-protected Rink Amide
MBHA resin on a 0.125 mmol scale, using appropriate
15   amino acids, palmitic acid and the compound from (a).
Coupling was carried out using standard TBTU/HOBt/DIEA
protocols. Simultaneous removal of the peptide from the
resin and deprotection of side-chain protecting groups
was carried out in TFA containing 5% EDT and 5% water
20   for 2 hours. Crude material was precipitated from ether
and purified by preparative liquid chromatography using
a gradient of 70 to 100% B over 60 minutes (A = 0.1%
TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate
.of 10 ml/min. After lyophilisation, a yield of 38 mg of
25   pure material was obtained (analytical HPLC, gradient
70-100% B over 20 minutes, A = 0.1% TFA/water and B =
0.1% TFA/acetonitrile, flow rate 1 ml/minute, detection
UV 214 nm, retention time 25 minutes). Further
characterisation was carried out using MALDI mass
30   spectrometry (ACH matrix), giving M+H at 1258, expected
1257.

c) Synthesis of N-[(S)-3-hexadecylthio-2-methylpropionyl]proline

35

DIEA (188 µl, 1.10 mmol) was added to a solution of 1-
iodohexadecane (176 mg, 0.500 mmol), captopril (120 mg,

0.550 mmol) and DBU (165 μl, 1.10 mmol) in
tetrahydrofuran (5 ml). The mixture was heated at 70 °C
for 2 hours and then concentrated. The residue was
poured onto water saturated with potassium hydrogen

5   sulphate and organic material was extracted into
chloroform. The organic phase was washed with water and
dried (MgSO$_4$). The product was purified by
chromatography (silica, CHCl$_3$/MeOH/AcOH 85:10:5) and
lyophilised to give 105 mg (48%) of white solid

10 material. The structure was verified by ^{1}H (500 Mhz) and
^{13}C (125 Mhz) NMR analysis and further characterised by
MALDI mass spectrometry, giving M-H in negative mode at
m/z 440 as expected.

15 d)   Preparation of gas-filled microbubbles comprising
DSPS, a lipopeptide containing atenolol and a lipophilic
derivative of captopril for diagnostic and therapeutic
applications

20   A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (4.5 mg) and products
from (b) (0.5 mg) and (c) in a vial. The mixture was
sonicated for 5 minutes and then heated at 80 °C for 5
minutes (vial was shaken during warming) and cooled.

25 The head space was flushed with perfluorobutane gas and
the vial was shaken in a cap mixer for 45 seconds,
whereafter the contents were extensively washed with
deionised water. MALDI mass spectrometry showed no
detectable level of compound from (b) or (c) in the

30 final wash solution. Incorporation of compounds (b) and
(c) into the microbubbles was confirmed by MALDI-MS as
follows: ca. 50 μl of microbubbles were transferred to a
clean vial containing ca. 100 μl of 90% methanol. The
mixture was sonicated for 30 seconds and analysed by

35 MALDI-MS (ACH-matrix), giving M+H peaks corresponding to
structures (b) and (c) respectively.

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e) *In vitro analysis*

The microbubbles were tested in the *in vitro* assay as
detected in Example 21. A gradual accumulation of
5   microbubbles binding to the cells was observed.

Example 63 - Gas-filled microbubbles comprising DSPS and
a cholesterol derivative of atenolol for diagnostic and
10   therapeutic applications

a) Synthesis of methyl 4-[(2,3-epoxy)propoxy]-
phenylacetate

15   A mixture of methyl 4-hydroxyphenylacetate (4.98 g,
0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and
pyridine (121 μl, 1.5 mmol) was stirred at 85 °C for 2
hours. The reaction mixture was cooled, and excess
epichlorohydrin was distilled off (rotavapor). The
20   residue was taken up in ethyl acetate, washed with brine
and dried (Na$_2$SO$_4$). The solution was filtered and
concentrated. The dark residue was chromatographed
(silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%)
of a colourless oil. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz)
25   spectra were in accordance with the structure.

b) Synthesis of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate

30   A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate
(2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol)
and water (1.35 ml, 74.7 mmol) was stirred at room
temperature overnight. The reaction mixture was
concentrated (rotavapor) and the oily residue was
35   dissolved in chloroform and dried (Na$_2$SO$_4$). Filtration
and concentration gave quantitative yield of a yellow
oil that was used in the next step without further

purification.   The structure was verified by ^{1}H and ^{13}C
NMR analysis.

c) Synthesis of 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid hydrochloride

A solution of methyl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetate (563 mg, 2.00 mmol) in
6M hydrochloric acid (15 ml) was heated at 100 °C for 4
hours.   The reaction mixture was concentrated
(rotavapor) and the residue was taken up in water and
lyophilised. ^{1}H and ^{13}C NMR spectra were in accordance
with the strucure and MALDI mass spectrometry gave a M+H
at 268 as expected.

d) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid

A solution of the 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid hydrochloride (2.0
mmol) in water (2 ml) was added to a solution of sodium
bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15
ml).   A solution of di-tert-butyl dicarbonate (0.48 g,
2.2 mmol) in dioxane (5 ml) was added.   Progress of the
reaction was monitored by TLC analysis (silica,
CHCl$_3$/MeOH/AcOH 85:10:5), and portions of di-tert-butyl
dicarbonate were added until conversion was complete.
The reaction mixture was poured onto water saturated
with potassium hydrogen sulphate and organic material
was extracted into ethyl acetate.   The organic phase was
washed with water and brine, dried (Na$_2$SO$_4$) and filtered
to give 0.6 g of crude material.   The product was
purified by chromatography (silica, CHCl$_3$/MeOH/AcOH
85:10:5).   The solution was concentrated and the residue
was taken up in glacial acetic acid and lyophilised.
Yield 415 mg (56%), white solid.   The structure was
confirmed by ^{1}H and ^{13}C NMR analysis.

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e)   Synthesis of cholesteryl N-Boc-β-alaninate

DIC (510 μl) was added to a solution of Boc-β-Ala-OH
(1.25 g, 6.60 mmol) in dichloromethane (15 ml) under an
inert atmosphere. The reaction mixture was stirred for
30 minutes and then transferred to a flask containing a
solution of cholesterol (1.16 g, 3.00 mmol) and DMAP
(367 mg, 3.00 mmol) in dichloromethane (15 ml). The
reaction mixture was stirred for 2 hours and then poured
onto an aqeous solution of potassium hydrogen sulphate.
After phase separation the aqueous phase was extracted
with chloroform. The combined organic phases were
washed with aqueous potassium hydrogen sulphate and
water and dried (MgSO₄). After filtration and
evaporation the crude product was chromatographed
(silica, chloroform/methanol 99:1) to give 1.63 g (97%)
of white solid. The structure was confirmed by ^{1}H NMR
(500 MHz).

f)   Synthesis of cholesteryl β-alaninate hydrochloride

A solution of compound from (a) (279 mg, 0.500 mmol) in
1M hydrochloric acid in 1,4-dioxane (5 ml) was stirred
at room temperature for 4 hours. The reaction mixture
was concentrated to give a quantitative yield of
cholesteryl β-alaninate hydrochloride. The structure
was confirmed by 1H NMR (500 MHz) analysis and by MALDI
mass spectrometry, giving a M+Na peak at 482, expected
481.

g)   Synthesis of cholesteryl N-Boc-4-[2-hydroxy-3-
[(1-methylethyl)amino]propoxy]phenylacetyl-β-alaninate.

To a solution of N-Boc-4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetic acid (55 mg, 0.15 mmol)
and cholesteryl β-alaninate hydrochloride (74 mg, 0.15

mmol) in DMF (5 ml) was added DIEA (26 ml, 0.15 mmol).
HOBt (23 mg, 0.15 mmol) and water-soluble carbodiimide
(WSC) (29 mg, 0.15 mmol) were added. The reaction
mixture was stirred at room temperature overnight and
then poured onto water (25 ml) containing sodium
carbonate (2.5 g) and sodium chloride (4.0 g).
Precipitated material was extracted into chloroform.
The organic phase was washed with water and dried
(MgSO$_4$). After filtration and concentration, crude
material (132 mg) was purified by column chromatography
(silica, chloroform/methanol/acetic acid, 95:4:1).
Pooled fractions were concentrated, taken up in glacial
acetic acid and lyophilised. Yield 83 mg (69%), yellow-
white solid. Structure was confirmed by ^{1}H NMR analysis.

h)   Synthesis of cholesteryl 4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetyl-ß-alaninate
trifluoroacetate

To a solution of N-Boc-4-[2-hydroxy-3-[(1-methyl-
ethyl)amino]propoxy]phenylacetyl-ß-alaninate (40 mg,
0.05 mmol) in dry dichloromethane (4 ml) was added
trifluoroacetic acid (2 ml). The reaction mixture was
stirred for 2 hours and then concentrated. The product
was lyophilised from a acetonitrile/water mixture to
give a quantitative yield of white-yellow material. The
product was characterised by MALDI mass spectrometry
giving M+H at 708 as expected.

i)   Preparation of gas-filled microbubbles comprising
DSPS and a cholesterol derivative of atenolol for
diagnostic and therapeutic applications

5   A solution of 1.4% propylene glycol/2.4% glycerol (1.0
       ml) was added to a mixture of DSPS (4.5 mg) and product
       from (h) (0.5 mg) in a vial. The mixture was sonicated
       for 5 minutes and then heated at 80 °C for 5 minutes
       (vial was shaken during warming) and cooled. The head
10   space was flushed with perfluorobutane gas and the vial
       was shaken in a cap mixer for 45 seconds, whereafter the
       contents were extensively washed with deionised water.
       MALDI mass spectrometry showed no detectable level of
       compound from (b) in the final wash solution.
15   Incorporation of compound from (h) into the microbubbles
       was confirmed by MALDI mass spectrometry.

j)   In vitro analysis

20   The microbubbles were tested in the in vitro assay as
       detailed in Example 21. A gradual accumulation of
       bubbles binding to the cells was observed.

25   Example 64 - Preparation of multiple-sepecific
       transferrin/avidin-coated gas-filled microbubbles for
       targeted ultrasound imaging

       This example is directed to the preparation of
30   microbubbles containing vectors for targeted
       ultrasound/therapy.

       a)   Synthesis of a thiol-functionalised lipid molecule:
       Dipalmitoyl-Lys-Lys-Lys-Aca-Cys.OH
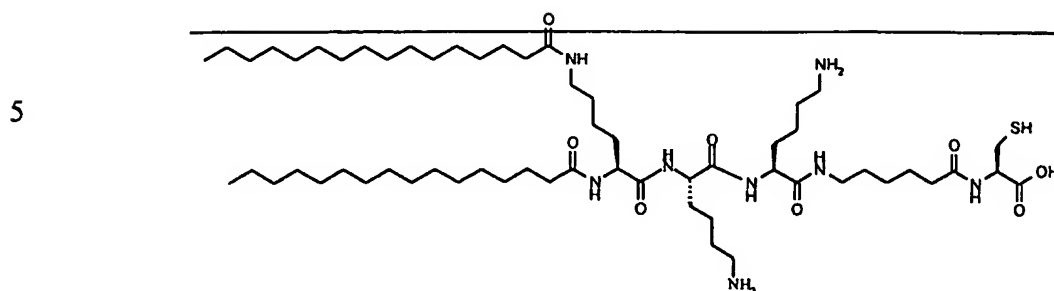
10 The lipid structure shown above was synthesised on an
ABI 433A automatic peptide synthesiser starting with
Fmoc-Cys(Trt)-Wang resin on a 0.25 mmol scale using 1
mmol amino acid cartridges. All amino acids and
palmitic acid were preactivated using HBTU coupling
15 chemistry. The simultaneous removal of peptide from the
resin and deprotection of side-chain protecting groups
was carried out in TFA containing 5% EDT and 5% H_2O for 2
hours, giving a crude product yield of 250 mg.
Purification by preparative HPLC of a 40 mg aliquot of
20 crude material was carried out using a gradient of 90 to
100% B over 50 minutes (A = 0.1 % TFA/water and B =
MeOH) at a flow rate of 9 ml/min. After lyophilisation,
24 mg of pure material was obtained (analytical HPLC,
gradient 70-100% B where B = 0.1% TFA/acetonitrile, A =
25 0.01% TFA/water: detection - UV 214 nm - product
retention time = 23 minutes). Further product
characterisation was carried out using MALDI mass
spectrometry: expected M+H at 1096, found at 1099.

30 b) Preparation of gas-containing microbubbles
comprising DSPS 'doped' with a thiol-containing lipid
structure

DSPS (4.5 mg) and the lipid structure from (a) above
35 (0.5 mg) were weighed into a clean vial and 0.8 ml of a
solution containing 1.4% propylene glycol/2.4% glycerol
in water was added. The mixture was warmed to 80°C for

5 minutes (vial shaken during warming) and filtered
while still hot through a 40 micron filter. The sample
was cooled to room temperature and the head space was
flushed with perfluorobutane gas. The vial was shaken
5   in a cap mixer for 45 seconds and then placed on aroller
table overnight. The resulting microbubbles were washed
several times with deionised water and analysed for
thiol group incorporation using Ellmans Reagent.

10   <u>c) Modification of transferrin and avidin with</u>
<u>fluorescein-NHS and Sulpho-SMPB</u>

To a mixture of 2 mg of transferrin (Holo, human) and 2
mg of avidin in PBS (1 ml) was added 0.5 ml of a DMSO
15   solution containing 1 mg Sulpho-SMPB and 0.5 mg
fluorescein-NHS. The mixture was stirred for 45 minutes
at room temperature then passed through a Sephadex 200
column using PBS as eluent. The protein fraction was
collected and stored at 4°C prior to use.
20

<u>d) Microbubble conjugation with modified</u>
<u>transferrin/aidin</u>

To the thiol-containing microbubbles from (b) was added
25   1 ml of the modified transferrin/avidin protein solution
from (c). After adjusting the pH of the solution to 9,
the conjugation reaction was allowed to proceed for 2
hours at room temperature. Following extensive washing
with deionised water the microbubbles were analysed by
30   Coulter counter (81% between 1 and 7 micron) and
fluorescence microscopy (highly fluorescent microbubbles
were observed).

35   <u>Example 65 - Gene transfer by gas-filled microbubbles</u>

This example is directed at the preparation of targeted

microbubbles for gene transfer.

a)   Preparation of gas-filled microbubbles comprising
DSPS and lipopeptide coated with poly-L-lysine

DSPS (4.5 mg) and lipopeptide from Example 41 (0.5 mg)
were weighed in two 2 ml vials. To each vial, 0.8 ml
propylene glycol/glycerol (4%) in water was added. Each
solution was heated at 80°C for 5 minutes, shaken and
then cooled to ambient temperature, whereafter the
headspaces were flushed with perfluorobutane. The vials
were shaken on a cap-mixer at 4450 oscillations/minute
for 45 seconds and put on a roller table for 5 minutes.
The content of the vials were mixed and the resulting
sample was washed by centrifugation at 2000 rpm for 5
minutes. The infranatant was removed and the same
volume of distilled water was added. The washing
procedure was repeated once. Poly-L-lysine HBr (20.6
mg) was dissolved in 2 ml water, then an aliquot (0.4
ml) was made up to 2 ml with water. To 1.2 ml of the
diluted poly-L-lysine solution was added 0.12 ml of the
DSPS-lipopeptide microbubble suspension. Following
incubation, excess polylysine was removed by extensive
washing with water.

b)   Transfection of cells

Endothelial cells (ECV 304) were cultured in 6 well
plates to a uniform subconfluent layer. A transfection
mixture consisting of 5 µg DNA (an Enhanced Green
Fluorescent Protein vector from CLONTECH) and 50 µl of
microbubble suspension from (a) in RPMI medium at a
final volume of 250 µl was prepared. The mixture was
left standing for 15 minutes at room temperature then 1
ml of complete RPMI medium was added. The medium was
removed from the cell culture dish and the DNA-
microbubble mixture was added to the cells. The cells

were incubated in a cell culture incubator (37 °C).

c)   Ultrasonic treatment

5   After 15 minutes incubation, selected wells were exposed
to continious wave ultrasound of 1 MHz, 0.5 W/cm², for 30
seconds.

d)   Incubation and examination

10

The cells were further incubated in the cell culture
incubator (37 °C) for approximately 4.5 hours. The
medium containing DNA-microbubbles was then removed by
aspiration, and 2 ml complete RPMI medium was added.
15   The cells were incubated for 40-70 hours before
examination. Most of the medium was then removed and
the cells were examined by fluorescence microscopy. The
results were compared to the results from control
experiments where DNA or DNA-polylysine were added to
20   the cells.

Example 66 - Flotation of endothelial cells by
microbubbles with vectors that specifically bind to the
25   endothelial cells

This experiment was carried out to show that the present
invention can be used for separation of cells to which
the microbubbles are targeted. The human endothelial
30   cell line ECV 304, derived from a normal umbilical cord
(ATCC CRL-1998) was cultured in Nunc culture flasks
(chutney 153732) in RPMI 1640 medium to which L-
glutamine (200 mM), penicillin/streptomycin (10,000 U/ml
and 10,00 μg/ml) and 10% fetal calf serum were added.
35   The cells were subcultured following trypsination with a
split ratio of 1:5 to 1:7 when reaching confluence.
2 million cells from trypsinated confluent cultures were

added to each set of five centrifuge tubes. Then
control microbubbles or microbubbles binding to
endothelial cells, made as described in Example 21 and
in Example 38, were added at 2, 4, 6 ,8 or 10 million
bubbles per tube. The cells at the bottom of the tubes
after centrifugation at 400 g for 5 minutes were counted
with a Coulter counter. It was found the 4 or more
microbubbles binding to a cell brought the cells to the
top of the fluid in the centrifugation tube. All cells
were floated by the microbbbles from Example 38 whereas
about 50% were floated with the microbubbles from
Example 21.

Example 67 - Gas-filled microbubbles of distearoyl-
phosphatidylserine comprising a lipopeptide containing a
vector with affinity for endothelin receptors for
targeted ultrasound imaging

a) Synthesis of 4'-[(3,4-dimethyl-5-isoxazolyl)-
sulfamoyl]succinanilic acid

To a solution of sulfisoxazole (267 mg, 1.00 mmol) in
DMF (10 ml) was added succinic anhydride (1.00 g, 10.0
mmol) and 4-dimethylaminopyridine (122 mg, 1.00 mmol).
The reaction mixture was stirred at 80°C for 2 hours and
then concentrated. The residue was taken up in 5%
aqueous sodium bicarbonate solution and extracted with
ethyl acetate. The aqueous solution was acidified with
dilute hydrochloric acid and organic material was
extracted into ethyl acetate. The organic phase was
washed with dilute hydrochloric acid, water and brine,
treated with active charcoal and dried (MgSO$_4$). The
solution was filtered and concentrated to give 280 mg
(76%) of white solid. The structure was verified by ^{1}H
(300 MHz) and ^{13}C (75 MHz) NMR spectroscopy. Further
characterisation was carried out using MALDI mass
spectrometry (ACH matrix), giving a M+Na peak at m/z 390

and a M+K peak at m/z 406 as expected.

b)   Synthesis of a lipopeptide functionalised with
sulfisoxazole
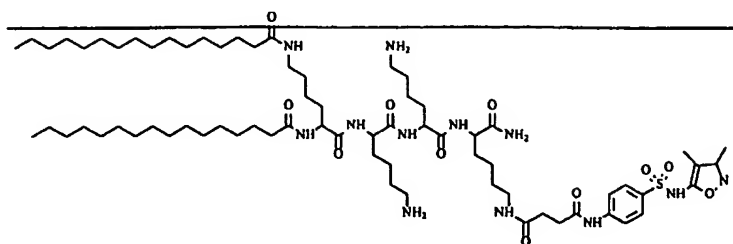
The structure shown above was synthesised on a manual
nitrogen bubbler apparatus starting with Fmoc-protected
Rink Amide BMHA resin on a 0.125 mmol scale, using
appropriate amino acids , palmitic acid and the compound
from (a). Coupling was carried out using standard
TBTU/HOBt/DIEA protocols. Simultaneous removal of the
peptide from the resin and deprotection of side-chain
protecting groups was carried out in TFA containing 5%
EDT and 5% water for 2 hours. Crude material was
precipitated from ether. The product was analysed by
analytical HPLC, gradient 70-100% B over 20 minutes, A =
0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate
1 ml/minute, detection UV 214 nm, retention time 27
minutes). Further characterisation was carried out
using MALDI mass spectrometry, giving a M+H at m/z 1359,
expected 1356.

c)   Preparation of gas-filled microbubbles comprising
the compound from (b)

A solution of 1.4% propylene glycol/2.4% glycerol (1.0
ml) was added to a mixture of DSPS (4.5 mg) and product
from (b) (0.5 mg) in a vial. The mixture was sonicated
for 5 minutes and then heated at 80°C for 5 minutes
(vial was shaken during warming) and cooled. The head

space was flushed with perfluorobutane gas and the vial
was shaken in a cap mixer for 45 seconds followed by
extensive washing with deionised water. MALDI mass
spectrometry showed no detectable level of compound from
5   (b) in the final wash solution. Incorporation of
isoxazole-containing lipopeptide into the microbubbles
was confirmed by MALDI-MS as follows: ca. 50 μl of
microbubbles were transferred to a clean vial containing
ca. 100 μl of 90% methanol. The mixture was sonicated
10   for 30 seconds and analysed by MALDI-MS (ACH-matrix),
giving a m+H peak at m/z 1359 corresponding to
lipopeptide (b).

15

Claims

1.   A targetable diagnostic and/or therapeutically
active agent comprising a suspension in an aqueous
carrier liquid of a reporter comprising gas-filled
microbubbles stabilised by monolayers of film-forming
surfactant, said agent further comprising at least one
vector.

2.   An agent as claimed in claim 1 wherein the gas
comprises air, nitrogen, oxygen, carbon dioxide,
hydrogen, an inert gas, a sulphur fluoride, selenium
hexafluoride, a low molecular weight hydrocarbon, a
ketone, an ester, a halogenated low molecular weight
hydrocarbon or a mixture of any of the foregoing.

3.   An agent as claimed in claim 2 wherein the gas
comprises a perfluorinated ketone, perfluorinated ether
or perfluorocarbon.

4.   An agent as claimed in claim 2 wherein the gas
comprises sulphur hexafluoride or a perfluoropropane,
perfluorobutane or perfluoropentane.

5.   An agent as claimed in any of the preceding claims
wherein the film-forming surfactant material comprises a
non-polymeric and non-polymerisable wall-forming
surfactant material, a polymer surfactant material or a
phospholipid.

6.   An agent as claimed in claim 5 wherein at least 75%
of the film-forming surfactant material comprises
phospholipid molecules individually bearing net overall
charge.

7.   An agent as claimed in claim 6 wherein at least 75%
of the film-forming surfactant material comprises one or

more phospholipids selected from phosphatidylserines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids and cardiolipins.

8.   An agent as claimed in claim 7 wherein at least 80% of said phospholipids comprise phosphatidylserines.

9.   An agent as claimed in any of the preceding claims wherein the film-forming surfactant material comprises a lipopeptide.

10.   An agent as claimed in any of the preceding claims wherein the vector is selected from antibodies; cell adhesion molecules; cell adhesion molecule receptors; cytokines; growth factors; peptide hormones and pieces thereof; non-peptide agonists/antagonists and non-bioactive binders of receptors for cell adhesion molecules, cytokines, growth factors and peptide hormones; oligonucleotides and modified oligonucleotides; DNA-binding drugs; protease substrates/inhibitors; molecules generated from combinatorial libraries; and small bioactive molecules.

11.   An agent as claimed in any of the preceding claims wherein the vector or vectors have affinity for targets at a level such that the agent interacts with but does not fixedly bind to said targets.

12.   An agent as claimed in claim 11 wherein the vector or vectors are selected from ligands for cell adhesion proteins and cell adhesion proteins which have corresponding ligands on endothelial cell surfaces.

13.   An agent as claimed in any of the preceding claims wherein the vector or vectors are sited such that they are not readily exposed to the target.

14.   An agent as claimed in any of the preceding claims
wherein the vector is covalently coupled or linked to
the reporter.

15.   An agent as claimed in any of claims 1 to 13
wherein the vector is coupled or linked to the reporter
through electrostatic charge interactions.

16.   An agent as claimed in any of claims 1 to 13
wherein the vector is coupled or linked to the reporter
by means of avidin-biotin and/or streptavidin-biotin
interactions.

17.   An agent as claimed in any of the preceding claims
which further contains moieties which are radioactive or
are effective as X-ray contrast agents, light imaging
probes or spin labels.

18.   An agent as claimed in any one of the preceding
claims further comprising a therapeutic compound.

19.   An agent as claimed in claim 18 wherein said
therapeutic compound is an antineoplastic agent, blood
product, biological response modifier, antifungal agent,
hormone or hormone analogue, vitamin, enzyme,
antiallergic agent, tissue factor inhibitor, platelet
inhibitor, coagulation protein target inhibitor, fibrin
formation inhibitor, fibrinolysis promoter,
antiangiogenic, circulatory drug, metabolic potentiator,
antitubercular, antiviral, vasodilator, antibiotic,
antiinflammatory, antiprotozoan, antirheumatic,
narcotic, opiate, cardiac glycoside, neuromuscular
blocker, sedative, local anaesthetic, general
anaesthetic or genetic material.

20.   An agent as claimed in claim 18 or claim 19 wherein
said therapeutic compound is covalently coupled or

linked to the reporter through disulphide groups.

21.   An agent as claimed in claim 18 or claim 19 wherein
a lipophilic or lipophilically-derivatised therapeutic
compound is linked to the surfactant monolayers
stabilising the gas-filled microbubbles of the reporter
through hydrophobic interactions.

22.   A combined formulation comprising:
      i) a first administrable composition comprising a
pre-targeting vector having affinity for a selected
target; and
      ii) a second administrable composition comprising
an agent as claimed in any of the preceding claims, said
agent comprising a vector having affinity for said pre-
targeting vector.

23.   A combined formulation as claimed in claim 22
wherein said pre-targeting vector is a monoclonal
antibody.

24.   A combined formulation comprising:
      i) a first administrable composition comprising an
agent as claimed in any of claims 1 to 21; and
      ii) a second administrable composition comprising a
substance capable of displacing or releasing said agent
from its target.

25.   A combined formulation comprising:
      i) a first administrable composition comprising an
agent as claimed in claim 20; and
      ii) a second administrable composition comprising a
reducing agent capable of reductively cleaving the
disulphide groups coupling or linking the therapeutic
compound and reporter in the agent of said first
administrable composition.

26. A process for the preparation of a targetable
diagnostic and/or therapeutically active agent as
defined in claim 1 which comprises either coupling or
linking at least one vector to a reporter comprising
gas-filled microbubbles stabilised by monolayers of
film-forming surfactant or generating gas-filled
reporter microbubbles using film-forming surfactant
having at least one vector attached thereto.

27. A process as claimed in claim 26 wherein a
therapeutic compound is also combined with the reporter.

28. A process as claimed in claim 27 wherein a
therapeutic compound containing thiol groups is linked
to thiol group-containing surfactant monolayers
stabilising the gas-filled microbubbles of the reporter
by reaction under oxidative conditions so as to generate
disulphide groups.

29. Use of an agent as claimed in any of claims 1 to 21
as a targetable ultrasound contrast agent.

30. A method of generating enhanced images of a human
or non-human animal body which comprises administering
to said body an agent as claimed in any of claims 1 to
21 and generating an ultrasound, magnetic resonance, X-
ray, radiographic or light image of at least a part of
said body.

31. A method as claimed in claim 30 which comprises the
steps:
        i) administering to said body a pre-targeting
vector having affinity for a selected target; and
thereafter
        ii) administering an agent as claimed in any of
claims 1 to 21, said agent comprising a vector having
affinity for said pre-targeting vector.

32.   A method as claimed in claim 31 wherein said pre-targeting vector is a monoclonal antibody.

33.   A method as claimed in claim 30 which comprises the
steps:
        i) administering to said body an agent as claimed
in any of claims 1 to 21; and thereafter
        ii) administering a substance capable of displacing
or releasing said agent from its target.

34.   A method as claimed in any of claims 30 to 33
wherein said agent further comprises a therapeutic
compound.

35.   A method as claimed in claim 34 wherein said
therapeutic compound is covalently coupled or linked to
the reporter through disulphide groups, and a
composition comprising a reducing agent capable of
reductively cleaving said disulphide groups is
subsequently administered.

36.   A method for *in vitro* investigation of targeting by
an agent as defined in any of claims 1 to 21 wherein
cells expressing a target are fixedly positioned in a
flow chamber, a suspension of said agent in a carrier
liquid is passed through said chamber, and binding of
said agent to said cells is examined.

37.   A method as claimed in claim 36 wherein the flow
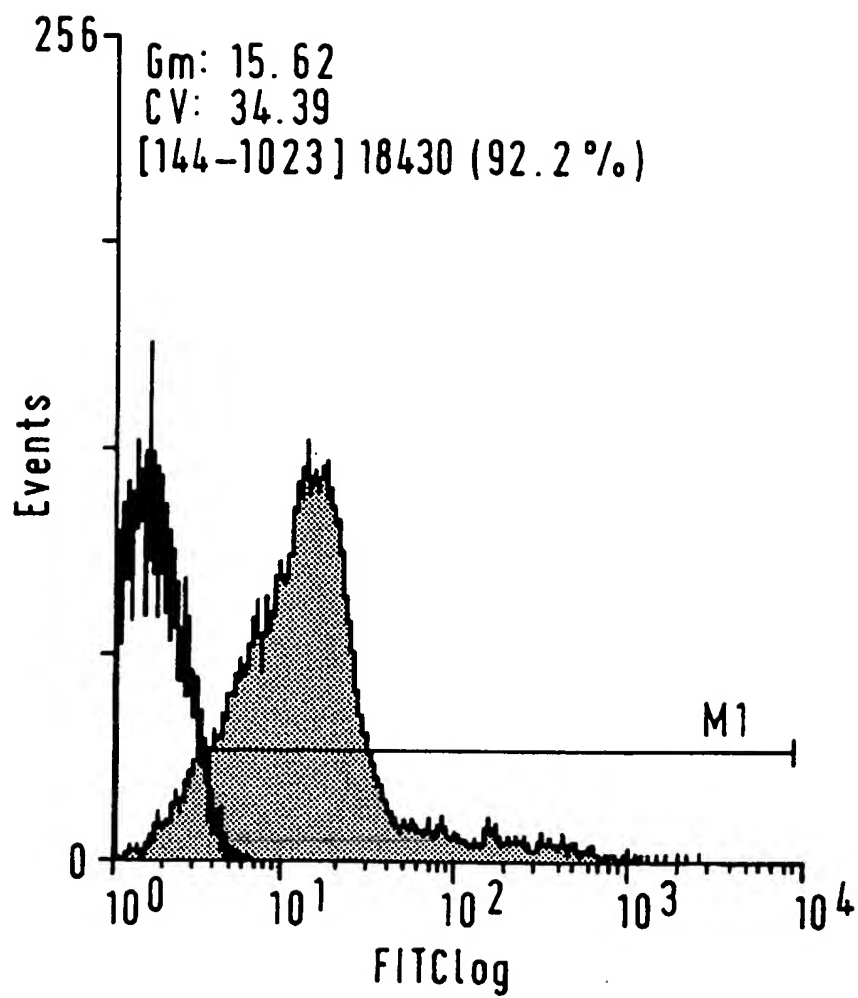rate of carrier liquid is controlled to simulate shear
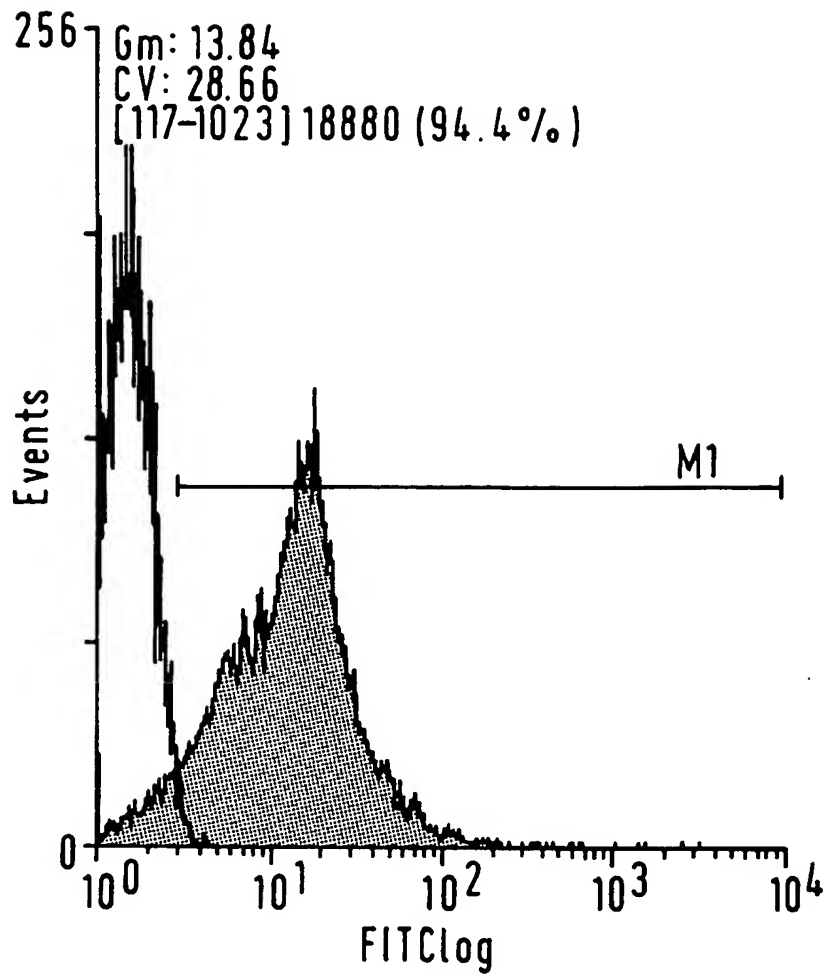rates encountered *in vivo*.

FIG. 1.

FIG. 2.